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THE EFFECT OF ANAEROBIC CONDITIONS ON TWO HETEROTRICH CILIATE PROTOZOA FROM PAPYRUS SWAMPS

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(*Received* 3 *April* 1959)

That a number of aquatic invertebrate animals can live for long periods in the complete absence of oxygen, and that many are actually called upon to do so in their natural habitat, is a reasonable conclusion to draw from the extensive work on this subject which has been reviewed by von Brand (1946). Since most animal tissues can derive energy from an anaerobic glycolysis, the major problem of adaption to prolonged lack of oxygen would appear to be the removal of (or acclimatization to) the products, mainly fatty acids, of an anaerobic metabolism. Many of the previous experiments were, however, open to criticism in view of the uncertainty that completely anaerobic conditions were attained. It is difficult also to establish beyond doubt that an animal has no access to traces of oxygen in nature, and the methods used for oxygen estimation were not likely to be sensitive to low concentrations in water containing reducing substances.

The experiments described here were carried out in the hopes of getting more precise information on the effects of anaerobic conditions on the survival of species of ciliate protozoa from papyrus swamps, using an improved method for oxygen estimation and at the same time measuring the oxidation-reduction potential in the water as an additional confirmation of anaerobic conditions when chemical analysis failed to detect oxygen.

The water in the interior of papyrus swamps is characteristically deficient in oxygen and is often completely anaerobic to within an inch of the surface (Beadle, 1932; Carter, 1954). But around the margins and in natural and cut clearings the water in pools may contain detectable or even abundant oxygen. A preliminary survey by one of us (J. R. N.) has shown, as might be expected, that certain protozoa are commonly found in the interior of the swamp and others are restricted to the better aerated water outside dense papyrus. The two species chosen for these experiments clearly differ in this respect and are both easily cultured in the laboratory.

MATERIALS

Both of the ciliates were collected from a papyrus swamp ten miles from Kampala on the Mubende road.

(1) *Bursaria* sp. (fam. Bursariidae). From examination of cultures now established in the Carlsberg Laboratory it is clear that this is a new species not yet described

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and is not *B. truncatella* as originally thought. It was found mainly in open unshaded pools and not in the interior of the swamps, and thus would appear to be sensitive to low concentrations of oxygen. It fluctuates greatly in numbers, and on one occasion in August 1955 there was a distinct whitish layer near the surface caused by the swarming of this ciliate.

(2) *Blepharisma undulans* (fam. Spirostomidae). This was common in water densely shaded by papyrus in which dissolved oxygen is often extremely low. It is peculiar in the possession of the pink photodynamic pigment 'Zoopurpurin' (Archikovskij, 1905), which disappears in the light and reappears in the dark. It is chemically related to the blue 'stentorin' found in *Stentor coeruleus*, both belonging to the meso-naphthodianthone group of compounds (Emerson, 1930*b*; Møller, 1958). Pigmented specimens are killed by strong light, but not in the absence of oxygen (Giese, 1946). Giese & Zeuthen (1949) found that strong light greatly increased the oxygen uptake of pigmented specimens before killing them, but only a slight increase was induced in specimens previously bleached in the light. In view of this apparent photo-oxidative action of the pigment two of the following experiments were designed to compare the resistance of pigmented with that of unpigmented forms to a low concentration of oxygen.

Both ciliates were cultured on agar plates 8–10 mm. deep in which sterile wheat grains were embedded and which were flooded with Pringsheim's salt solution* to a depth of 3–4 cm. The plates were inoculated with the ciliates, together with some unsterilized swamp water from which grew the organisms serving as food. The *Bursaria* cultures were kept on a shelf in dull light. The culture medium for *Blepharisma* was prepared and inoculated in the same way, but the pH was adjusted to 8·0 which was found by Giese (1953) to be the optimum. The cultures were kept in the dark. When colourless specimens were needed cultures were kept for several days on the open bench, though not in the direct light. The colourless *Blepharisma* in the lighted cultures were rather smaller and more slender than the pigmented forms in the darkened cultures, the latter containing some of the giant forms noted by Giese (1946). Subcultures were made every 2 or 3 weeks.

METHODS

For each experiment 4 or 5 l. of swamp water containing a small amount of fine organic debris and inoculated with ciliates were stirred vigorously in an aspirator jar while being run into five or more pairs of 200 ml. glass-stoppered reagent bottles. This was done to ensure that each bottle contained the same number of ciliates and amount of suspended organic matter as well as the same initial concentration of oxygen. The bottles were filled to the top, stoppered without including air and kept under water. In experiments with pigmented *Blepharisma* the bottles were kept in the dark; with *Bursaria* and colourless *Blepharisma* they were exposed to dull light during the day.

* K_2HPO_4, 0·02 g.; NaCl, 0·02 g.; $Ca(NO_3).4H_2O$, 0·20 g.; $MgSO_4.7H_2O$, 0·02 g.; $FeSO_4.7H_2O$, 0·002 g.; water, 1 l.

Once daily the condition of the ciliates was noted after examination through a hand-lens, and a pair of bottles was opened, one for estimation of oxygen, the other for measurement of pH and oxidation-reduction potential.

Dissolved oxygen was estimated by an adaption of the double-iodine modification of the Winkler method involving a preliminary clarification with aluminium hydroxide (Beadle, 1958). With waters of oxidation-reduction potential too low to be consistent with the presence of dissolved oxygen this method may give small negative values for oxygen content.

For oxidation-reduction potential a bright platinum electrode was lowered to near the bottom of the second sample of the pair which was connected to a saturated calomel reference electrode through a KCI-agar bridge. The pH was then measured by substituting a glass electrode for the platinum electrode. A Cambridge pH meter was used for both measurements. The pH ranged from 6·1 to 7·9, and a correction was applied to the measured oxidation-reduction potential to adjust the figure to pH 7·0 (E_7), and the potential of the reference electrode was corrected for temperature. Reasonably steady potentials were obtained after 5 min. and the readings were taken after 10 min. The final measurements in each experiment were made on the day on which the ciliates were found to have died. The temperature in the laboratory during all the experiments was 22–25° C. In the course of any one experiment it would have ranged over less than 2° C.

With the object of destroying all organisms other than those introduced with the inoculation from the cultures, all the experiments were repeated using swamp water which had previously been boiled for about 10 min. and then aerated. In this way it was hoped both to retard the processes leading to anaerobic conditions and perhaps to alter the nature of the chemical reactions involved. If, in spite of this, the survival of the ciliates bore the same relation to oxygen concentration as in the experiments with unboiled water, the conclusion that oxygen is the primary factor determining survival would be more justified, though by no means certain.

Each experiment involving several pairs of bottles and lasting several days was repeated three to nine times. The three curves reproduced in each figure are the two most divergent together with one intermediate curve from each set of experiments.

RESULTS

Bursaria sp. (Figs. 1A, B)

Without exception the ciliates were always dead on the day on which the oxygen concentration approached zero. This happened also in the experiments using boiled water, in which the oxidation-reduction potential did not drop as much as in two at least of the experiments using unboiled water. In both sets however anaerobic conditions were reached in 3 days.

Blepharisma undulans. Pigmented form (Fig. 2A, B)

The experiments showed clearly and without exception that this species will withstand 1–3 days of anaerobiosis. This survival period does not seem to be influenced either by the length of time taken for the oxygen to be exhausted or by

the course or level of the oxidation-reduction potential curve. Considerable changes in these were produced by previous boiling of the water.

Blepharisma undulans. Colourless form (Fig. 3A, B)

The absence of the pigment made no significant difference to the survival in oxygen-free water. Previous boiling of the water did not retard the onset of anaerobic conditions and the results of the two sets of experiments were remarkably similar.

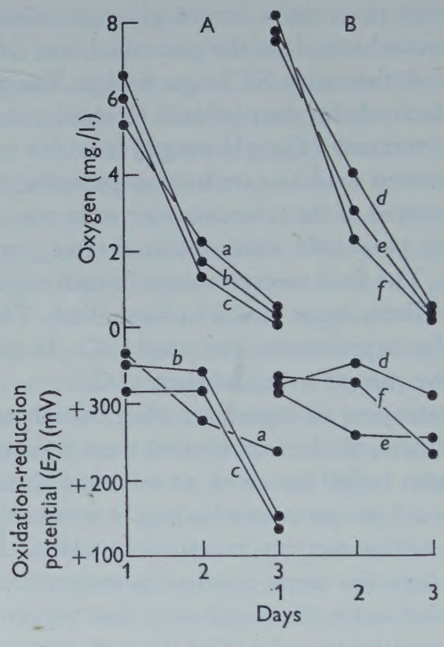

Fig. 1. *Bursaria* sp. A. Unboiled, aerated swamp water. Three of five experiments. B. Previously boiled and aerated swamp water. Three experiments in all. pH, start to finish: *a*, 7·3–6·9; *b*, 7·3–6·5; *c*, 6·3–6·2; *d*, 7·9–6·6; *e*, 7·9–6·7; *f*, 7·5–6·7.

DISCUSSION

Bursaria sp. is therefore incapable of surviving under anaerobic conditions whilst *Blepharisma undulans* will live up to 3 days without measurable oxygen.

The oxidation-reduction potential curves from the experiments with *Bursaria* (Fig. 1) show that the final potential (E_7) could be as high as +300 mV (B, *d*) or as low as +125 mV (A, *c*). In one experiment the *Blepharisma* died when the potential was +300 mV (Fig. 2 B, *e*), and in another it had dropped to −190 mV (Fig. 2A, *c*). The changes in pH of the medium were in all cases relatively small and occurred in both directions, and moreover did not depart from the range normally found in natural swamp water.

It seems, therefore, that the concentration of dissolved oxygen is the primary factor involved and that *Bursaria* is incapable of an anaerobic metabolism. This was further supported by two of the experiments on *Blepharisma* in which some

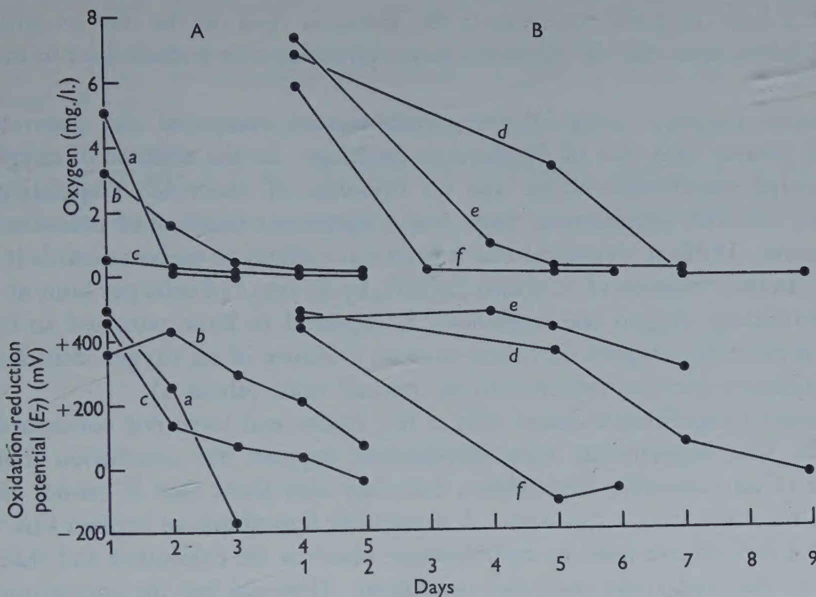

Fig. 2. *Blepharisma undulans* (pigmented). A. Unboiled, aerated swamp water. Three of nine experiments. B. Previously boiled and aerated swamp water. Three of four experiments. pH, start to finish: *a*, 6·0–6·6; *b*, 6·3–6·5; *c*, 6·2–7·0; *d*, 7·7–7·7; *e*, 7·5–6·8; *f*, 7·2–6·8.

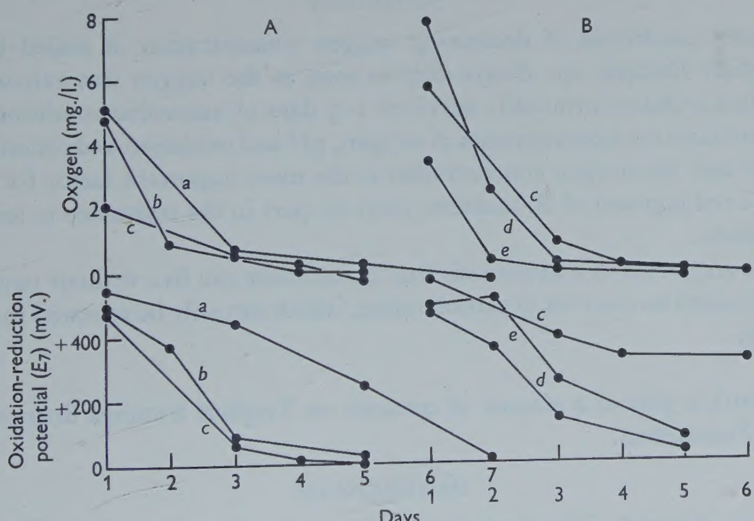

Fig. 3. *Blepharisma undulans* (colourless). A. Unboiled, aerated swamp water. Three of four experiments. B. Previously boiled and aerated swamp water. Three of four experiments. pH, start to finish: *a*, 6·8–6·7; *b*, 6·1–6·6; *c*, 6·1–6·8; *d*, 7·3–6·7; *e*, 7·3–6·6; *f*, 6·7–6·4.

Bursaria were accidentally introduced in the swamp water. One of these is shown in Fig. 2 A, *a*. In both experiments the *Bursaria* died on the day on which the oxygen fell to zero, but the *Blepharisma* survived for 1 or 2 more days in the same water.

Emerson (1930*a*), using Warburg manometers, compared the respiration of *Amoeba proteus* with that of *Blepharisma undulans*. In the absence of oxygen and with added bicarbonate there was no evidence of anaerobic respiration with *Amoeba*, but with *Blepharisma* there was a significant increase of pressure in the manometer. If this is assumed to have been due entirely to carbon dioxide it would amount to the evolution of 12·5 mm.^{3} of CO_2 by 80 mm.^{3} of cells per hour at 20° C. On readmitting oxygen the respiration is reported to have returned to normal, which presumably implies that there was no evidence of an oxygen debt and that the respiratory quotient returned to the normal value (about 1).

Emerson's experiments lasted only a few hours and were not concerned with survival. Our experiments with *Blepharisma* support the conclusion that it is capable of an anaerobic metabolism, but they also show that it cannot thereby survive for more than a few days. A reasonable hypothesis to explain this would be that it can survive until its carbohydrate reserves are exhausted and that these cannot be renewed under anaerobic conditions. However low the concentration of oxygen in the natural habitat it would always be possible for it to have occasional access to oxygen in the surface film.

SUMMARY

1. Under conditions of decreasing oxygen concentration in sealed bottles of swamp water *Bursaria* sp. always died as soon as the oxygen was exhausted, but *Blepharisma undulans* invariably survived 1–3 days of anaerobic conditions.

2. Simultaneous measurements of oxygen, pH and oxidation-reduction potential suggested that the oxygen concentration is the most important factor for survival.

3. The red pigment of *B. undulans* plays no part in the resistance to low oxygen concentration.

4. It is suggested as a hypothesis that *B. undulans* can live without oxygen until it has exhausted its reserves of carbohydrate, which can only be renewed in presence of oxygen.

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A TOUCH-LEARNING CENTRE IN *OCTOPUS*

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INTRODUCTION

Octopuses blinded by section of their optic nerves can be trained to discriminate between the members of pairs of objects touched (Wells & Wells, 1956, 1957*a*). Removal of the optic lobes—more than half of the total mass of the brain—does not affect their performance in such discriminations (Wells & Wells, 1957*b*), but removal of a comparatively small amount of tissue from the vertical lobe, lying on top of the central supraoesophageal brain mass, produces deficiencies that may be broadly described as decreasing the efficiency of the learning process; after operation a greater number of trials is required to reach prescribed standards of response in training experiments. The nature of the deficit caused by vertical lobe removal has been considered for tactile discriminations by Wells & Wells (1957*b*, *c*, 1958*a*, *b*) and for visual discriminations by Boycott & Young (1955, 1957).

The present account deals with the results of supraoesophageal lesions that extend beyond the optic-vertical lobe system and include the basal lobes. The latter form the greater part of the central supraoesophageal brain mass and are known to be concerned with the integration of movements of the animal as a whole (Boycott & Young, 1950, 1960); the part they play in learning cannot be investigated using visual techniques comparable with those used to investigate vertical lobe function (Boycott, 1954) since their removal upsets motor co-ordination and prevents the animal from attacking or retreating from things seen. The problem has therefore been approached using tactile training techniques involving movements of only one arm at a time that are co-ordinated within the arm concerned. It will be shown that while the basal lobes play no part in learning to recognize things touched, the areas immediately anterior to them, the inferior frontal and subfrontal lobes, are essential; without them an octopus cannot be taught to discriminate between objects that it touches.

MATERIAL

Octopuses of between 250 and 1000 g. from the Bay of Naples were obtained and treated as described by Boycott (1954). Before use in training experiments the animals were blinded by section of the optic nerves and most of them were subjected to a further operation in which parts of the brain were cut out. Details of operational methods are given in Wells & Wells (1956, 1957*b*). Individual animals are identified in this account by the number with which they were labelled in the original protocols. This label consists of a prefix B, C or D showing the year

(1955–7) in which the experiment was carried out, a serial number, and suffixes indicating the lesion or lesions made. The suffixes that concern us here are B = blind (optic nerves cut), NOL = no optic lobes, i.e. the optic lobes removed after section of the optic stalks peripheral to the optic glands (Wells & Wells, 1957*b*), NV = vertical lobe removed, NSF = superior frontal lobe removed and NB = basal lobes removed. Animals having still larger lesions including the basal lobes and extending forward into the inferior frontal lobe system are indicated NB+. The relative position of these parts is shown in Fig. 1.

Typical basal lobe lesions (NB animals) included removal of all parts of the central supraoesophageal brain mass behind the inferior frontal and subfrontal lobes, while vertical lobe removals (NV animals) extended only sufficiently far into the subvertical and dorsal basal lobes to ensure complete vertical lobe excision. In most cases the superior frontal lobe was also removed to ensure 100% removal of the vertical lobe, which is otherwise technically difficult because a part of it underlies the upper part of the superior frontal. There appears to be no difference in the quality of performance of NV animals with and without the superior frontal lobe in tactile experiments (Wells & Wells, 1957*b*, 1958*a*, *b*).

ANATOMY AND METHOD OF PLOTTING LESIONS

After training experiments the brains of operated animals were fixed and lesions checked from serial sections prepared using the modification 'B' of Cajal's silver method given in Sereni & Young (1932). Lesions are plotted on a standard diagram representing a median longitudinal section through the supraoesophageal lobes of the brain (Fig. 1*c*). Most of the lesions made can be classed as belonging to one of the seven types illustrated in Fig. 2; the smallest lesions (type *A*) are incomplete vertical lobe removals, the rest (types *B* to *G*) include the vertical lobe and extend progressively further into the supraoesophageal brain. It will be shown from training experiments that there is essentially no difference in the quality of performance of animals having lesions of types *B* to *F*, which range from removals of the vertical lobe (but nothing else) to removals including the vertical, superior frontal and all of the basal lobes (type *F*). The same is not true, however, of lesions encroaching upon the inferior frontal and subfrontal lobes, and here more detailed mapping is necessary and an additional section at right angles to the standard longitudinal diagram is used (Fig. 1*e*). This transverse section passes vertically through the inferior frontal at the level at which the sensory tracts from the arms enter the supraoesophageal lobes. These tracts pass around the oesophagus and divide as they reach the upper part of the brain. The outer branches lead into the median part of the inferior frontal lobe; the inner branches run into the lateral parts of the inferior frontal and from here many of the nerves continue upwards and backwards to the subvertical lobe (Fig. 1*d*). It will be observed that lesions of type *D* deprive the hind part of the brain of this source of sensory input, while types *A*, *B* and *C* do not affect it. Degeneration experiments show that the great majority of the sensory nerves in these tracts run directly from the arms into the

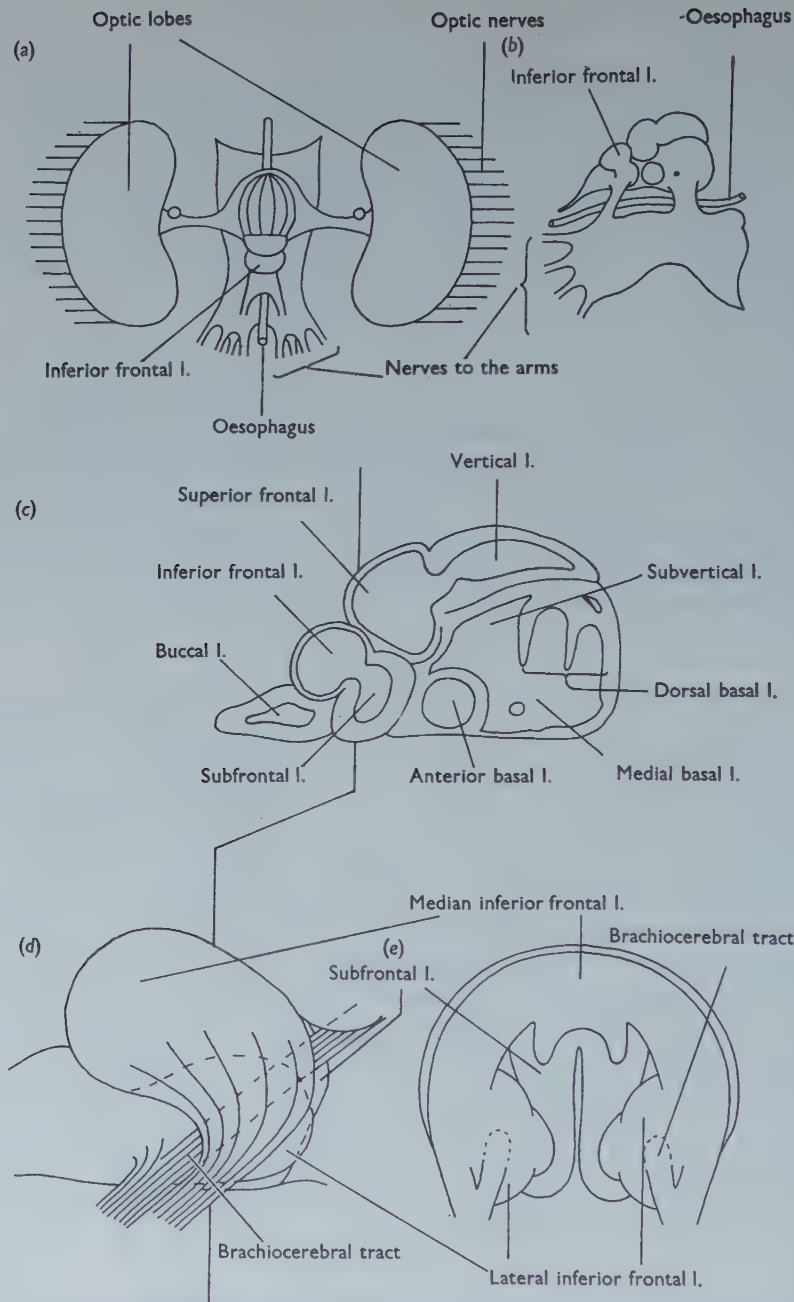

Fig. 1. The brain of *Octopus* and divisions of it mentioned in the text. (*a*) shows the brain from above as it would be seen after removal of the cartilage surrounding the central mass. (*b*) is a vertical longitudinal section through this. (*c*) is a longitudinal section through the supra-oesophageal lobes only, and is used in this form as a standard diagram on which lesions are plotted in Figs. 2–5. In an animal of 500 g. this would be about 4 mm. long. (*d*) and (*e*) give further details of the inferior frontal/subfrontal region and its relation to the brachiocerebral tracts: (*d*) being a view from the left side, and (*e*) a transverse section at the level shown on (*d*). (*e*) is also used as a standard diagram for plotting lesions in Figs. 3 and 5. In the text the complex formed by median inferior frontal, lateral inferior frontal and subfrontal lobes is referred to as the 'inferior frontal system'.

supraoesophageal lobes without synapse in the lower centres of the brain (Boycott & Young, 1960).

The standard transverse section also cuts through the subfrontal lobe. This region is structurally rather unlike the rest of the inferior frontal,* being composed of a great many very small cells (about $5 \cdot 2 \times 10^6$—ten times as many as in the whole of the rest of the inferior frontal (Young, 1960)) forming thick walls around a neuropil of fine processes. In Cajal silver preparations the nuclei of these cells stain a characteristic reddish colour (as do the small cells of the vertical lobe) in contrast to the larger neurones of the rest of the brain, which become yellow, brown or black. The neuropil of the subfrontal is divided into two lateral halves linked by extensive commissures in a plane parallel with and immediately behind the section shown in Fig. 1*e*. In the standard longitudinal diagram the subfrontal is shown as if cut slightly to one side of the mid-line, and in Figs. 3–5 the condition on the least damaged side is shown.

There are extensive connections between both the lateral and median inferior frontal and subfrontal lobes. The lateral inferior frontal is broadly continuous with the buccal and is defined as beginning where the buccal neuropil becomes divided into two lateral halves, at approximately the same vertical level as the anterior edge of the median inferior frontal (Fig. 1*d*). Further details of the structure and connexions of these parts are given in Boycott & Young (1960).

In a few cases lesions so large as to include the whole of the inferior frontal and subfrontal lobes were made. Such animals can eat if the buccal lobe remains substantially intact (see below) but do not learn to recognize objects by touch. Details of these very large lesions (*G* in Fig. 2, and greater) are not plotted.

ESTIMATES OF THE PROPORTION OF LATERAL INFERIOR FRONTAL AND SUBFRONTAL LOBES REMAINING AFTER OPERATION

It will be shown below that the extent of lesions to the subfrontal/lateral inferior frontal system is extremely critical; the proportion of tissue remaining cannot be shown sufficiently accurately on standard diagrams and measurements of the amount of tissue remaining, calculated from serial sections, are given in Table 4.

The figures for the proportion of lateral inferior frontal remaining were obtained as follows: the number of sections on which the buccal lobe neuropil occurred was counted; this gives a measure, independent of the size of the animal concerned, for the number of sections on which the lateral inferior frontal might be expected to appear if complete, since the two series are equally long in controls. The lateral inferior frontal on each side of each section was then examined and scored on a 4-point scale as 1, $\frac{3}{4}$, $\frac{1}{2}$ or $\frac{1}{4}$ present and the total score for each side divided by the number of sections on which buccal neuropil occurred. This figure ($\times 100$) is the estimated percentage of lateral inferior frontal present quoted in Table 4. Because the figure depends upon subjective estimates of the proportion of intact

* In decapod cephalopods there is no subfrontal lobe (Thore, 1939); in octopods the subfrontal is broadly continuous above and laterally with the inferior frontal lobe and presumed to be derived from it (Boycott & Young, 1960.)

tissue in each section, and because there is inevitably some distortion of the brain when extensive lesions are made, the final percentage may be in error by as much as 20% and as an estimate of the number of cells involved will therefore be accurate only to within ± 6000 cells (the lateral inferior frontal is estimated to contain 30,000 nerve cells in each of its lateral halves; this and other cell counts from intact animals are taken from Young (1960)).

Estimation of the amount of subfrontal tissue remaining was more difficult; here the proportion of the lobe remaining in animals that learned was commonly less than 5% of the whole, and an attempt was made to count the actual number of cells left, it being assumed that all those that could be seen were functional. Here the method was to count the number of nuclei in a unit area in a section of standard thickness and estimate the total number on an area basis. The density of the cells in the areas remaining was not, however, constant, and although allowance was made for this, the counts (summarized in Table 4) are reliable only as giving the order of number of units involved (see p. 609).

It was assumed for present purposes that all of the experimental animals had the same number of cells in their lateral inferior frontal and subfrontal lobes before operation, an assumption that is, strictly speaking, only valid if cells are not added to the system as the animal grows through the 250–1000 g. size range used. It is known that the volume of certain parts of the brain of *Octopus* (notably the vertical lobe, which, like the subfrontal lobe, is composed of a great many very small cells) increases with respect to the rest of the brain during post-embryonic development (Wirz, 1954), but it is not known whether this represents an increase in the number of cells or in the size of individual cells already present.

THE EFFECT OF LESIONS ON POSTURE AND EXPERIMENTS WITH BRAINLESS PREPARATIONS

A preliminary survey of the defects caused by removal of parts from the supra-oesophageal lobes has been published by Boycott & Young (1950), and a much more detailed study is in preparation (Boycott & Young, 1960). Briefly, removal of the vertical, superior frontal and optic lobes produces no defects in the movements of blind octopuses, but damage to the basal lobes upsets motor co-ordination so that the animals cannot 'walk' or swim in a normal integrated manner and tend to sit on the floor of their tanks with the arms in confusion. None of this, however, affects the nature of the movements that the individual arms make in grasping and taking or rejecting objects touched. Even preparations consisting only of the ring of eight arms connected by the interbrachial web will regularly perform all the movements necessary to transfer small objects from the armtips to the mouth, provided that the test is carried out within about 10 min. of decapitation. Thus in a typical series of experiments ten out of thirteen such preparations grasped and passed pieces of sardine to the mouth (or, rather, to where the mouth had been) with the first arm to be tested. Four of these animals had been killed because they had ceased to feed and immediately before decapitation had persistently rejected bits of sardine exactly similar to those taken readily after removal of the brain.

As yet no means of eliciting rejection movements with equal regularity has been discovered, but occasionally brainless preparations do make complete and unmistakable rejection movements, thrusting objects away to arms length, and it must be supposed that these, too, are integrated within the nerve cord of the arm concerned. The observation that it is much easier to elicit taking than rejection movements is significant in view of the rest of the experimental results to be reported, which show an unusually high proportion of positive responses by animals with very large lesions.

THE EFFECT OF LESIONS ON FEEDING AND THE ABILITY OF ANIMALS TO EAT REWARDS GIVEN

Removal of the buccal lobe cuts the nerve supply to the beak and octopuses with extensive lesions in this region cannot eat. Animals with the buccal lobe substantially intact, but lacking the greater part of the inferior frontal system, can eat sardines, but often have difficulty with more complex food such as crabs, which are taken and passed to the mouth as usual, but remain held there alive for hours or days. Normal octopuses kill crabs with a secretion from the posterior salivary glands within a minute or so of taking them (Lo Bianco, 1909), and dismember the dead crabs generally without breaking up the endophragmal skeleton. Animals with only the buccal lobe, or with the buccal and parts of the lateral inferior frontal lobes remaining, may sometimes kill their crabs, but they seem to do so by tearing them apart. They are then unable to eat the contents, or at best devour only a part of the crab, leaving fragments—the gills, for example—that would normally be eaten, and breaking up the endophragmal skeleton (see Table 1, where such cases are indicated +/−).

The posterior salivary glands are innervated from the buccal lobe (Pfefferkorn, 1915; see also Bacq & Ghiretti, 1953), and the failure to kill crabs in a normal manner may be due to a failure to secrete poison, perhaps because of interference with the normal sensory input to the buccal lobe from the arms. It is also possible that the failure is attributable not so much to an inability to secrete poison as to an inability to manoeuvre the crab into a position suitable for its injection. At present there are no data available that would enable one to decide between these alternatives, other than the observation, first made by Lo Bianco (1909), that it is generally impossible to detect any break in the cuticle of crabs removed, dead, within a few minutes of seizure by an octopus; this implies that elaborate manipulation is not necessary to kill crabs, though it may well be necessary to eat them efficiently.

EXPERIMENTAL METHODS

Perspex cylinders 3·0 cm. long and 2·5 cm. in diameter were used as test objects. One of these, P1, had narrow longitudinal grooves cut into it; the other, P4, remained smooth (see Fig. 4). Two types of experiments were made using these objects, as follows:

(1) Experiments in which animals were taught to reject a given object—P1 or P4—repeatedly presented to them at short intervals (generally 5 min.; a few of the

experiments were made with tests at 3 min. intervals without apparent effect upon the results obtained). The normal untrained reaction of a healthy octopus is to grasp and pass to the mouth any small unfamiliar object that it touches; beak marks on soft objects such as lumps of cork or plasticine indicate that the animal tries to eat things that it takes in this way. If given a small electric shock (6 V. a.c. by means of electrodes fixed to a probe with which the animal can be touched under water) for taking the test object, an octopus speedily learns to reject the object by thrusting it away whenever it is presented. The performance of individuals can be compared in terms of the number of errors made (and shocks received) before an object presented is consistently rejected.

In these and in discrimination experiments the test object was always presented to the same arm of the octopus under training, and shocks given on the web immediately above this arm when the object was taken; this is because extensive lesions are liable to cause differences in the behaviour of the two sides of the same animal, and because learning by the individual arms is to some extent independent, a matter that is discussed elsewhere (Wells, 1959).

(2) Discrimination experiments. In these the animals were trained to take one of the two test objects whenever it was presented and to reject the other. Animals were rewarded with pieces of sardine for taking one (the 'positive') of the two objects, and punished with an electric shock, as described above, for taking the other (the 'negative' object). One object (+ or −) was presented at each trial, and there were forty trials per day in two groups of 20. The trials in each group of twenty were systematized thus + − + − + + − − + − + − − + + − + − + −, individual trials within a group being at 5 min. intervals; the start of the second daily group of trials was not less than 6 hr. after the start of the first. Animals were trained until they made 75% or (more commonly) 85% correct responses in a group of twenty trials (5 errors or less and 3 errors or less respectively). The performance of individuals can be compared in terms of the number of groups of trials required to train them to these prescribed standards of accuracy of response.

EXPERIMENTAL RESULTS

A. *Repeated presentation experiments, with shocks for acceptance*

If given a weak electric shock each time that it takes a particular object presented to it, a blind but otherwise unoperated octopus learns to reject the object within three or four trials. In the present experiments each individual series was continued with successive presentations, always to the same arm of the animal, until the object had been rejected for a number of times (generally 6 times) in succession, whereupon the object was presented to an arm on the other side of the animal and tests continued on that side. When such a change is made in the course of experiments with trials at short intervals (as here), the second arm to be tested will at first take the test object, only ceasing to do so after one or more shocks have been received—the animal apparently has to relearn to reject the test object after the change. The phenomenon is fully discussed elsewhere (Wells, 1959), and here

Table 1. *Responses to the same object (P 1 or P 4) presented at 5 min. intervals*

(The octopus was given a 6 V. a.c. shock if it took the object after examining it. + = Positive response, animal [pass]ed the object to the mouth (and got a shock); − = negative response, animal examined the object and withdrew, or rejected the object by thrusting it away.)

Animal	Trials		Feedings	
			Ate sardines	Ate crabs
	Controls			
97 B	+ − + − − − − − − −		.	.
99 B	+ + + − − − − − − −		.	.
119 NOL	+ − + − − − − − + + − − −		.	.
120 NOL	+ + − − − − − − −		.	.
121 B*	+ + − − − − − −		.	.
123 B*	+ − + − − − − − −		.	.
127 NOL	+ + + + − − − − − −		.	.
	Vertical lobe removed (Lesion types *C* and *D*)			
89 NVNSFNOL	+ + + + + + + + + + + + + + − − + − − − − − − − −		.	.
91 NVNSFNOL	+ + + + − − − − − − − − + + − − − − − − − − − − −		.	.
112 NVNSFNOL	+ − − − − − − − − −		.	.
113 NVNSFNOL	+ + + + + + + − − + + − − − − −		.	.
143 NVB	+ + + + − − − − − − − − − − − − − − − −		.	.
	Basal lobes removed (Lesion types *E* and *F*)			
121 NBB*	+ + − − − − − − − −		+	+
123 NBB*	+ + − − − − − −		+	+
119 NBNOL	+ + + + − − + + − − − − − − − − + − − −		+	NR
121 NBNOL	+ + − − − − − − − −		+	NR
138 NBNOL	+ − − − − − − − − −		+	NR
	Basal lobes removed with damage to the inferior frontal lobe			
122 NB+B (RHS)	− + + + + + + + + + + + + + +		+	+/−
124 NB+B (RHS)	+ + + − − − + + + + + + + + +		+	+/−
131 NB+NOL (RHS)	+ − − − − − −		+	+
126 NB+NOL (RHS)	+ + − + − − − − −	Octopuses with asymetrical lesions. B 127, B 128 and B 132 were afterwards subjected to a long series of tests (Fig. 2) and learned on one side	+	+
(LHS)	+ + + + + + + + + + + + +		.	.
127 NB+NOL (LHS)	+ + + + + + + +		+	+
(RHS)	− + + + + + + −		.	.
128 NB+NOL (LHS)	+ + + + + + + + + + + + +		+	+
(RHS)	+ + + + + + +		.	.
132 NB+NOL (LHS)	+ + + − + + + + + + − + +		+	+/−
(RHS)	+ + + + + + +		.	.
117 NB+NOL (RHS)	+ + + + + + + + + + + + + + + + + + + +		+	+
	All supraoesophageal lobes removed except the buccal lobe (Lesion type *G*)			
118	+ + + + + + + + + + + + + + + + + + + +		+	−
	All supraoesophageal lobes removed			
117	+ + + + + + + + + + + + + +		−	−
38	+ + + + + + − + + + + + + + + + + + − −		−	NR
39	+ + + + + + + + + + + + + − + − + + + +		−	NR

* B 121 and B 123 appear twice, having been tested before and after operation. Other animals had no pre-operational experience of the test objects. In the majority of cases similar results were obtained in tests with the right [and] left sides of the animal, and only the results with the first side to be tested are given here. + and − in the ['F]eedings' columns indicate (respectively) that the animal did or did not eat the food specified. +/− = sometimes (see p. 595) and NR = no record.

Table 1 lists only the performance of the first arm to be tested up to the trial immediately preceding a change of arm. Subsequent sets of trials are recorded only in cases when the two sides of the same animal behaved in a markedly different manner as, for example, when an octopus learned using the first arm to be tested but failed to show any capacity for learning when training was continued using an arm on the contra-lateral side. After the last trial of each training experiment the octopus was given a piece of sardine; if it failed to take this the test was considered invalid and the results discarded.

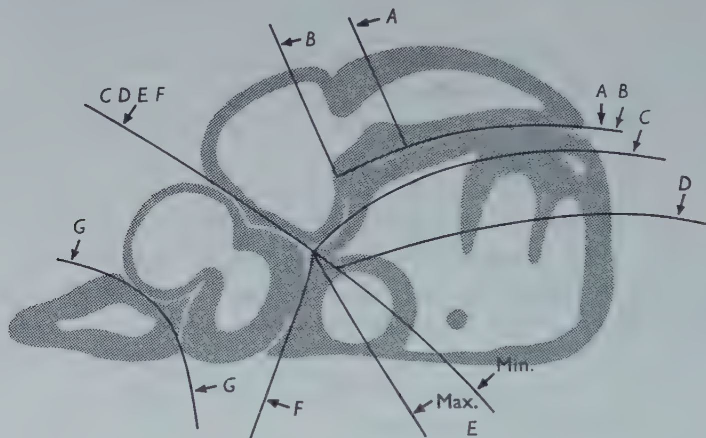

Fig. 2. Types of lesion made. Most of the lesions made in the experimental animals can conveniently be classified as belonging to one of the 7 types shown here; the lesion in each case includes all of the area shown above and behind the demarcation line. Removal of the greater part of the vertical lobe (lesions type *A*) increases the number of errors that octopuses make in learning to discriminate between objects by touch. The proportion of errors is further increased by extension of the lesion to include the whole of the vertical lobe (type *B*), but there is no further decline in performance when additional tissue is removed in lesions of types *C–F*. In contrast to this animals with type *G* lesions cannot learn at all. The inferior frontal region is critical for touch learning and lesions encroaching upon these areas are plotted individually in Figs. 3 and 5.

The majority of the results fall into two clear categories. Animals either learned quickly to reject the object presented, within three or four trials, or they failed to learn altogether. Five of the seven controls made only 2 errors each before settling down to a steady rejection of the test object, and the remaining two controls made 3 and 4 errors respectively (Table 1). Seven out of the ten animals with lesions to the vertical and basal lobes performed as well as controls, learning to reject the test object within the first four trials. The other three took six, nine and fifteen trials to learn to make the same response to the same object; these were *not* the animals with the largest lesions, indeed the two worst performances (those of B89 and B113, Table 1) were by animals having relatively small lesions, not including the basal lobes. It seems probable from this that the variation in the number of trials required by animals with the vertical and basal lobes damaged depends upon individual pre-experimental experience with objects touched rather than upon the

extent of the lesions made, and this view is supported by the observation that, in the present sample, the average number of errors made by the five animals with the basal as well as the vertical lobes removed (i.e. those with the five largest lesions) was not significantly different from that of controls (experimentals mean 2·6, controls 2·4).

The performance of animals having lesions that included damage to the inferior frontal system was quite different from that of animals with lesions limited to the vertical and basal lobes. When the inferior frontal was removed altogether (*G*, Fig. 2), octopuses could no longer be taught to reject an object repeatedly presented to them (Table 1).

Between this extreme and the essentially normal performance of animals with the vertical and basal lobes removed fall the performances of individuals in which tactile learning was impaired to a greater or lesser extent as a result of lesions encroaching upon, but not entirely destroying the inferior frontal system. These range from normal performances by octopuses such as B 126 (RHS) and B 131, that learned as quickly as controls, to complete failures to learn by B 122, B 124 and C 117 Maps of the lesions in these animals are included in Fig. 5. The experiments with B 126 and B 131 show that simple tactile learning to reject an object repeatedly presented to a specified arm can proceed after removal of the median inferior frontal (B 131) and after removal of the entire inferior frontal system from the contralateral side (B 126, RHS, – the LHS, as might be expected, did not learn). None of the remaining six animals with damage to the inferior frontal system behaved normally and three of them (B 122, B 124 and C 117) continued to take the test object, without any observable change in behaviour, for as long as tests were continued. The other three (B 127, B 128 and B 132), however, began to show signs of hesitation after the first few trials, examining the object for an unusually long time before taking it, or starting to reject the object when it was presented before finally passing it under the web to the mouth. It was considered worth while extending the series of tests with these animals, and the results of the extended tests are plotted in Fig. 3, together with maps of the lesions in the animals concerned. It can be seen that each of these octopuses learned better on one side than on the other when both sides were tested and that in all three cases this was the side where the lesion was less extensive (see Table 4 for details of the amount of tissue left on the two sides in these animals). Once again the results indicate that the median inferior frontal is not an essential part of the touch-learning apparatus since both B 128 and B 132 learned without it (but see p. 609).

It is more difficult to assess the importance of the lateral and subfrontal divisions of the inferior frontal system. None of the animals learned after complete removal of the subfrontal lobe, which suggests that this region plays some important part in the process of learning to reject objects by touch, an implication that is not surprising since more than nine-tenths of the nerve cells in the inferior frontal system are subfrontal. The subfrontal cells are for the most part very small and even the relatively insignificant fragments of the subfrontal lobe left after the more extensive operations contained a large number of cells compared with the intact

lateral inferior frontal; thus B127 (RHS), for example, with 0·7% of the subfrontal and 55% of the lateral inferior frontal intact had more subfrontal than lateral inferior frontal cells left (Table 4). As a result of the very great disparity in the number of cells in the two parts, and because it is impossible to remove the lateral

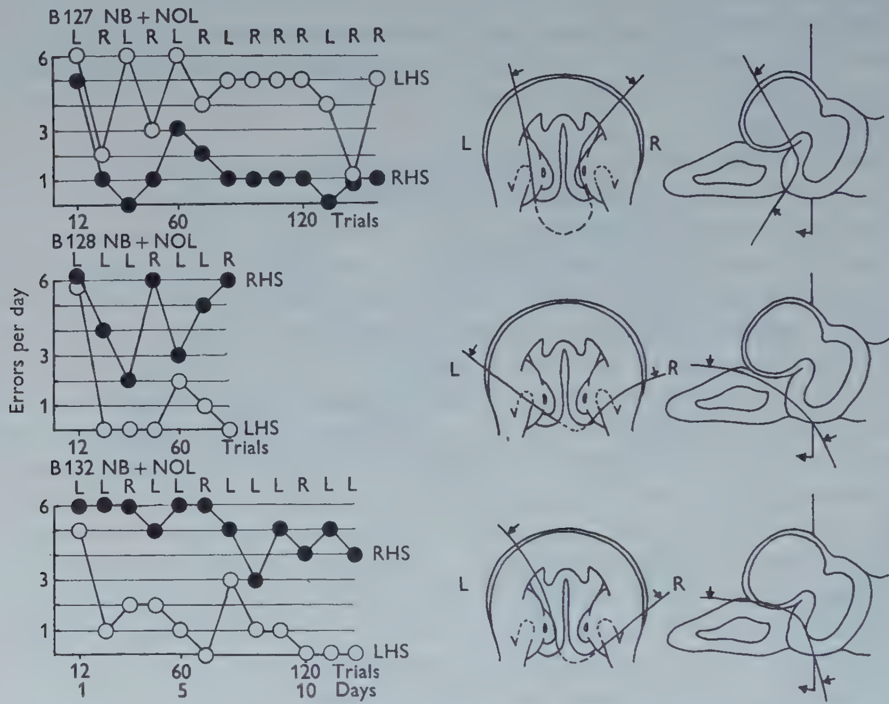

Fig. 3. Results of training three octopuses with lesions extending into the inferior frontal system. There were 12 trials per day (rather more on the first day, see Table 1—only the result with the first six is plotted here), 6 with each side of the animal, first one and then the other side being tested in a single series of trials at five minute intervals. At each trial the same object was presented and the octopus given a 6 V. a.c. shock if it took it. The performances of the two sides of each animal are plotted separately, ○ being that of the second left arm, and ● that of the second right; the first side to be tested on any particular day is indicated L or R respectively. Estimates of the actual number of lateral inferior frontal and subfrontal cells left in these animals are given in Table 4. Note that over a period of days, as here, a drop in the number of times that an object is taken in daily tests does not necessarily imply learning; a number of other factors can influence the probability of a positive response (see, for example, the effect of a surgical operation upon the performance of C54 in Fig. 4), so that, strictly speaking these results show only that the 'better' side learned and give no indication of the capacity of the other (for a further discussion of factors affecting the probability of positive responses in this sort of experiment see Wells (1959)).

inferior frontal without cutting the principal output channel from the subfrontal, we cannot find out from the present type of experiment whether the two parts have distinct functions in touch learning. Certainly the proportion of lateral inferior frontal remaining (within the limits 50–100%—more extensive damage interferes with the output from the subfrontal) does not appear to have any effect upon the

outcome of training experiments, whereas the presence or absence of subfrontal tissue does, but this could be due to the considerable increase in the total number of cells left when subfrontal tissue remains, and does not necessarily imply a special function for the subfrontal. The question of subdivision of function within the inferior frontal system is further discussed on page 608.

Table 2. *A summary of the performance of octopuses trained to discriminate between the two Perspex objects P1 and P4.*

'he table gives details of experiments not already published elsewhere, and not included in Fig. 4; the summary 'able 3 includes the performances of animals already quoted in Wells & Wells (1957*b*, 1958*a*).)

Animal	*Errors per group of 20 Trials* (10+, 10−).
	Controls*
156 B	*7* 7 **1** 4 1 0
158 B	9 *4* **2** 0 1 0
186 B	10 10 8 *5* 6 5 **2** 5 1 1 0 1 2 0
187 B	6 **3** 2 0
11 B	*4* 6 **2** 1 0 (RT 4)
16 B	*4* **2** 0 1 (RT 3)
19 B	6 **2** 0 0 (RT 2)
33 B	10 *5* **0** 1 1 0 (RT 1)
	Vertical lobe 85 % removed (Lesion type *A*)
144 NVB	6 **3** 2 1
	Vertical lobe removed (lesion types *B*, *C* and *D*)*
167 NVNSFNOL	7 *5* **3** 4 Reversal 14 10 *4* **2** 5 0 4 1
37 NVNSFB	10 *5* 6 5 **2** 3 1 0 (RT 3)
41 NVNSFB	10 9 10 10 10 8 7 *5* 6 **3** 2 1 1 2 2 0 1 1 1 1 (RT 2)
49 NVNSFB	10 10 10 10 10 6 7 *4* 5 **2** 3 3 3 1 0 1 1 2 0 5 (RT 8)
51 NVB	10 9 *4* 4 **3** 3 1 0 0 2
	Basal lobes removed (lesion types *E* and *F*)*
146 NBNOL	11 6 10 6 **2** 2 5 2 Rev. 10 *7* 7 **3** 1 3 1 (RT 1)
60 NBB	11 7 7 *5* 7 6 5 **0** 0 0 0
121 NBNOL	10 10 *7* **2** 1 0 0 0 (RT 5)
	Basal lobes removed with damage to the inferior frontal lobe*
171 NB+NOL (RHS)	*5* 5 4 4 5 8 5 **2** Rev. 11 13 13 14 16 10 11 10 14 12 11 11 9†
(LHS)	11 15 12 11 12 10 10 13 13 11 10 11
117 NB+NOL (RHS)	10 10 12 10 10 11 11 12 10 10
40 NB+NOL (RHS)	9 8 8 9 8 8 10 8 10 10
43 NB+B (RHS)	*7* 10 5 5 11 6 9 9 11 12
(LHS)	10 10 9 12 7 10 12 8
47 NB+B (RHS)	9 *7* 10 8 6 6 5 9 7 6
(LHS)	*4* 6 **2** 0 2 6
49 NB+B (RHS)	10 13 10 6 7 7 9 10 8 12 11
50 NB+B (RHS)	9 *5* 8 7 5
(LHS)	8 10 12 13

Maps of these lesions (43, 47, 49 and 50 NB+B) are given in Fig. 5

A further example from each of these groups is given in Fig. 4.
Fig. 4, plot of the further performance of B171 follows on here.
'T = Retention test, without rewards or punishments, carried out after a 5-day break in training. Reversal = ning in which the originally positive object was used as the negative and vice versa, following on the initial ning after a break of 36 hr. Figures in italic (5) and black (**3**) show when the animal first made respectively 75 % 85 % correct responses (or better) in a group of 20 trials.

Whatever the individual rôles of the subfrontal and lateral inferior frontal, there can be no doubt that these regions between them include the site of whatever organizational changes accompany the alteration from positive to negative responses when the animal learns to reject an object touched. If these parts remain, octopuses can be taught to reject objects touched; if they are removed the animals fail to learn.

Repeated presentation experiments such as those outlined above reveal no changes in capacity to alter responses towards objects touched, following removal of the basal lobes and the parts overlying them. This is in contrast to the finding from discrimination experiments that vertical lobe removal increases the number of trials required to train octopuses to given standards of accuracy of response (Wells & Wells, 1957*a*, *b*, 1958*a*—see also Table 3). It appears that experiments involving a succession of negative trials at short intervals are insufficiently sensitive to detect small differences in capacity to learn, and it is therefore necessary to use slightly more elaborate techniques, setting the animals somewhat more difficult problems to resolve, in order to evaluate the effect of removal of the vertical and basal lobes. The results with one such technique are discussed below.

B. *Discrimination experiments*

Animals were trained to discriminate between P 1 and P 4 as described on p. 596. This is rather an easy discrimination for *Octopus*, and control animals made 75 % or more correct responses in the second or third group of twenty trials; by the fourth group most individuals made better than 85 % correct responses.

Octopuses with the vertical lobe removed (lesion types *B–D*) took about twice as many trials to achieve the same standards of accuracy of response under the same conditions. Fourteen such animals averaged 4·0 groups (the figure for each individual includes the group in which it first attained the prescribed standard of accuracy) to attain a standard of 75 % correct responses, compared with an average of 2·3 groups by twenty-six controls. Training was continued with ten of the animals from which the vertical lobe had been removed, and these averaged 5·6 groups to reach the 85 % standard of accuracy of response, a performance that may be compared with that of 24 controls averaging 3·5 groups (Table 3—details of individual performances that have not already been published elsewhere are given in Table 2 and in Fig. 4).

Most of the results with animals having still larger lesions fall into one or another of two distinct categories, as in the simpler experiments involving repeated presentations of the same object, but again it is the few results that do not quite fit into either category that are most interesting. The performance of four animals in which the lesions included the basal lobes without encroaching upon the inferior frontal system (lesion types *E* and *F* in Fig. 2) was not appreciably different from that of animals lacking only the vertical lobe. The number of trials required in initial training, reversal of responses, and the score in retention tests all fell well within the range shown by animals having smaller lesions of types *B* to *D*. Thus the four animals (B 146, C 54, C 60 and C 121) in which the lesions included the basal lobes averaged 4·3 and 5·8 groups of twenty trials to reach the 75 and 85 % standards of

Table 3. *A summary of the performance of octopuses after lesions impairing but not preventing learning in discrimination experiments*

(Each entry under 'Results' shows the number of groups of 20 trials required to attain the stated standards of accuracy of response, these results being listed in the same order as the animals in each column.)

		Animals with lesions of increasing extent					
	Controls	Vertical lobe removed				Vertical and basal lobes removed	
		A	*B*	*C*	*D*	*E*	*F*
Animals concerned	26 blind octopuses without central lesions	B162 B164 C144	C11	B158 B161 C9 C10 C16 C18 C41 C51	B167 C37 C45 C49	B146 C121	C54 C60
Results No. o groups of trials to reach criterion 75 % correct responses	Range 1–4	5 5 2	4	7 5 3 1 4 1 8 3	2 2 4 8	5 4	4 4
	Mean 2·35	4·0	4·0	4·0	4·0	4·5	4·0
			Mean for 100 % vertical lobe removals = 4·0			Mean for basal lobe removals = 4·25	
85 % correct responses	Range 1–7	— 5 2	4	7 5 3 — — — 10 5	3 5 4 10	5 4	6 8
	Mean 3·46	3·5	4·0	6·0	5·5	4·5	7·0
			Mean for 100 % vertical lobe removals = 5·6			Mean for basal lobe removals = 5·75	

'*t*' tests confirm that the difference in performance of controls and 100 % vertical lobe removals is significant, and that the difference between 100 % vertical lobe removals and basal lobe removals is not.

A = 80–90 % of the vertical lobe removed; *B* = vertical lobe entirely removed but the superior frontal lobe substantially intact; *C* = superior frontal removed as well as the vertical, but brachio-cerebral tracts not disconnected from the subvertical; *D* = lesion extends into the subvertical and probably disconnects the brachio-cerebral tracts; *E* = posterior basal lobe removed as well as the vertical, but parts of the anterior basal remain; *F* = all basal lobes removed. Maps of these types of lesion are given in Fig. 2.

The detailed performance of most of these animals (all those not listed in Table 2 or Fig. 4) has already been recorded in full in Wells & Wells (1957*b* or 1958*a*).

response, figures that are not significantly different from the 4·0 and 5·6 groups of trials required by animals lacking only the vertical lobe (Table 3). Learned responses to the test objects were reversed in one of the NB animals (B 146 NBNOL) which made 85 % correct responses in the 4th group of twenty trials after reversal. The same animal, C 54 and C 121 were also given retention tests, making 1, 5 and

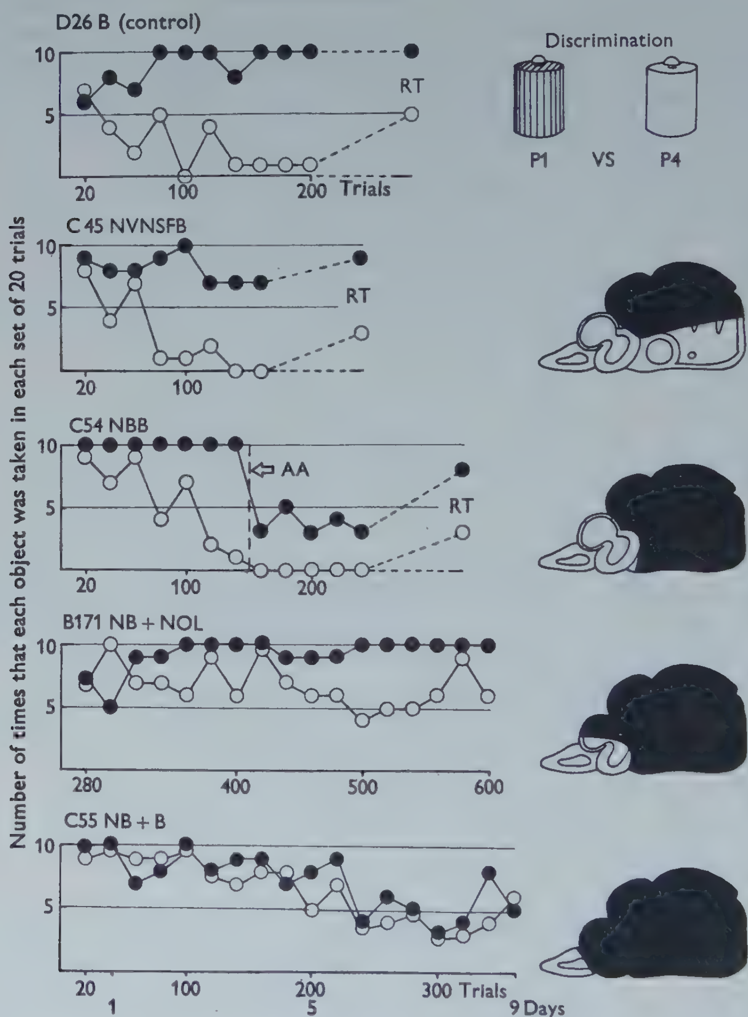

Fig. 4. The effect of lesions upon the performance of octopuses in discrimination experiments, showing that while removal of the basal lobes does not seriously upset the performance of a tactile discrimination, damage to the inferior frontal system does. ●, Positive object; ○, Negative object. The plots show the number of times that each object was taken in each group of 20 trials (10+, 10−), there being two such groups per day. Where two points coincide, because the number of positive and negative objects taken was the same, these are displaced up- and downwards respectively. RT = Retention test, carried out after a break in training of 5 days; AA (in the plot of C54NBB) = arm amputated (not that used in training). The performance of B 171 recorded here follows on that given in Table 2; further details of the lesion in this animal are given in Fig. 5 and Table 4.

5 errors respectively in the twenty-trial tests (carried out as in training but without rewards or punishments) 5 days after the end of training. C121 was also tested after a further 10 days, and on this occasion made 3 errors only. All of these figures are well within the range of results obtained with animals lacking only the vertical lobe (Wells & Wells, 1957*b*, 1958*b*) and, unless it be supposed that all four animals in the present sample were exceptional individuals, this can only mean that the basal lobes play no part in learning to respond differentially to objects touched—there is no additional effect upon tactile learning when they are removed as well as the vertical lobe. The performances of controls, of animals with the vertical lobe removed, and of animals with the basal lobes as well as the vertical removed are summarized and compared in Table 3. Details of the performance of typical individuals, showing that errors are made predominantly by acceptance of the negative object in all three groups, are given in Fig. 4.

Compared with that of animals having the basal lobes removed the performance of octopuses with slightly larger lesions, encroaching upon the inferior frontal system, was exceedingly poor. The animals with the two largest lesions in this group (C117, D40) did not learn at all and continued to take the test objects indiscriminately for as long as training was continued; these octopuses had parts of the lateral inferior frontal only remaining in addition to the buccal lobe. (A single animal, C55, with the buccal lobe left but all parts of the inferior frontal system removed also failed to learn.) Other octopuses, with a little more of the inferior frontal system remaining performed only slightly better (Table 2, Fig. 4). The extent of the lesion in this area is so critical that it is often necessary to consider the left and right sides of each animal separately as in the 'repeated negatives' experiments, and in Table 2 this is done for four animals in which the lesions made (Fig. 5) were unequal on the two sides.

Table 2 shows that some, at least, of the animals with extensive lesions to the inferior frontal system were able to discriminate between objects using the arms on one side or the other; thus B171 (RHS), D47 (LHS) and D50 (RHS) all made 75% or better correct responses in more than one group of twenty trials during training. Others, such as D43 (LHS), D49 (RHS) and D50 (LHS) showed no evidence of ability to distinguish between the objects during the 80–200 trials to which they were subjected. These results can be correlated with the lesions made, the octopuses—or sides of octopuses—that discriminated having substantially more cells of the subfrontal/lateral inferior frontal region intact than those that did not (Table 4).

In one of the animals that successfully discriminated, B171 (in which these responses were also, eventually, reversed by further training—Table 2 and Fig. 4) the median inferior frontal was almost wholly removed, so that the possibility of an input channel from it to the subfrontal was eliminated; this would appear to confirm the finding from repeated negative experiments that the median inferior frontal does not constitute an essential link in the tactile learning process. It is, however, significant that when a change was made from right to left the left side was found to be quite unaffected by the intensive training given to the right,

and still persisted in the performance initially shown by the right side (see p. 609).

The remaining results are less easy to assess. D 43 (RHS) and D 47 (RHS) both made 75% correct responses in one or more groups of trials in the course of training, but in neither case was this standard maintained, and although both appeared to discriminate between the objects to some extent no further improvement in performance resulted when training was continued. The performance of D 47 (RHS) was, if anything, rather better than that of D 43 (RHS) and this again is in keeping with the lesions made, that in D 43 being considerably the greater of the two (Fig. 5 and Table 4).

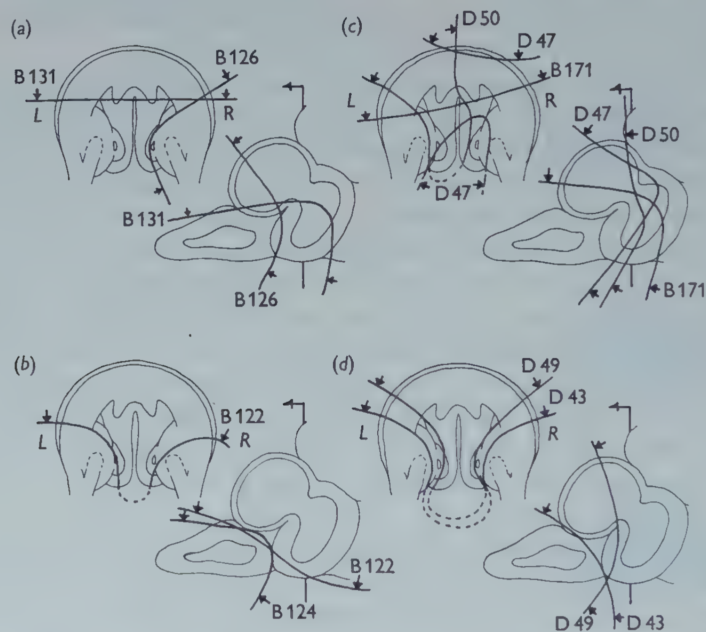

Fig. 5. Lesions in animals from Tables 1 and 2 not plotted elsewhere. (*a*) Lesions in two animals that learned to recognize an object repeatedly presented (RHS—B 126 did not learn with the LHS); and (*b*) lesions in two that failed to learn (RHS tested) under the same conditions. (*c*) Lesions in three animals that learned to discriminate between the objects P 1 and P 4 (B 171, D 50—RHS learned, LHS did not—and D 47—LHS learned, RHS (see text, p. 606); and (*d*) lesions in two animals that failed to discriminate (D 43, both sides, D 49 RHS only tested). Lesions in two further animals, D 40 and C 117, neither of which learned, were more extensive than that in D 43 and have not been plotted.

It will be noticed that every one of the animals that learned to discriminate had a considerable proportion of the subfrontal lobe intact as well as the greater part of the lateral inferior frontal. Thus in D 47 (LHS), D 50 (RHS) and B 171 (both sides) a total of approximately 1×10^6 nerve cells from these lobes remained; D 47 (RHS), with about one-third of this number, learned only slowly; and D 49 (RHS), with 39,000, not at all. These figures are all greater than those associated with normal or almost normal performance in the repeated presentation

Table 4. *Proportions of lateral inferior frontal and subfrontal lobes remaining*

(The two sides of each animal are treated separately and the percentage figures given show the proportion remaining of tissue normally present on one side. 100% = 30,000 lat. inf. fr. cells and 2,600,000 subfrontal cells. Performances of these animals in training experiments are summarized in Tables 1 and 2 and in Fig. 3; maps of the lesions are given in Figs. 3 and 5.)

Animal	Side	Subfrontal remaining	Lat. inf. fr. remaining	Total cells left	Remarks
		A. Repeated presentation of negative object experiments			
B131	R	60% $>1.5\times10^6$ cells	85% 25,400 cells	$>1.5\times10^6$	Learned (one mistake)
B126	R	0.5% 12,800 cells	85% 25,400 cells	38,200	Learned (three mistakes)
	L	Nil	Nil	Nil	No learning
B132	L	1.6% 41,200 cells	100% 30,000 cells	71,200	Learned slowly
	R	Nil	20% 6,000 cells	6,000	No learning
B128	L	0.8% 21,700 cells	80% 34,000 cells	45,700	Learned slowly
	R	Nil	30% 9,000 cells	9,000	No learning
B127	R	0.7% 17,000 cells	55% 16,400 cells	34,000	Learned slowly
	L	0.05% 1,300 cells	45% 13,200 cells	14,500	No learning
B122	R	0.5% 12,800 cells	70% 21,000 cells	33,800	No learning
C117*	R	Nil	65% 19,500 cells	19,500	No learning
B124	R	Nil	35% 10,500 cells	10,500	No learning
		B. Discrimination experiments			
B171	R	60%+ $>1.5\times10^6$ cells	100% 30,000 cells	$>1.5\times10^6$	Learned
D50	R	36% 926,600 cells	95% 28,400 cells	955,000	Learned
	L	Nil	95% 28,400 cells	28,400	No learning
D47	L	35% 901,000 cells	85% 25,400 cells	926,400	Learned
	R	13% 342,000 cells	85% 25,400 cells	367,400	Learned slowly
D49	R	0.6% 15,000 cells	80% 24,000 cells	39,000	No learning
D34	R	Nil	80% 24,000 cells	24,000	No learning
	L	Nil	80% 24,000 cells	24,000	No learning
C117*	R	Nil	65% 19,500 cells	19,500	No learning
D40	R	Nil	20% 6,000 cells	6,000	No learning

* C117 appears twice, having been used in both types of experiment.

experiments, where animals such as B 126 (RHS) and B 127 (RHS) learned to reject an object after lesions leaving a total (lateral inferior frontal + subfrontal) of less than 39,000 cells (Table 4). Evidently a greater number of nerve cells is required if the animal is to resolve differences between two more or less similar objects like P 1 and P 4 than when it is merely required to learn to reject either of these while continuing to accept the pieces of sardine on which it is fed.

In these experiments it is again impossible to assess the importance of the lateral inferior frontal separately from that of the subfrontal, although it is significant that the lateral inferior frontal remained intact in nearly all of the animals, including some of those that failed to learn. The possibility of attributing separate functions to the lateral and subfrontal subdivisions of the inferior frontal system is further discussed below.

DISCUSSION

The first object of the experiments reported in this account was to find out which structures in the brain of the octopus play a part in learning to recognize objects by touch. The function of the vertical, optic and superior frontal lobes in this respect has already been surveyed elsewhere (Wells & Wells, 1957*b*, *c*; 1958*a*, *b*). It was shown that of these regions only the vertical lobe played any part in tactile learning and that this contributed by amplifying the effect of each trial. It is not itself essential to the process, however, and learning to recognize objects by touch proceeds (though more slowly) without it, in some other part of the brain. The experiments that have been reported here show that the part concerned is the inferior frontal system, since this is the only region of the brain demonstrably essential to the process of learning to recognize objects by touch. Unless a proportion of it is left, responses towards objects touched remain unchanged, even when training is continued for many times the number of trials found necessary in controls. In contrast to this area, where a small lesion can produce a marked difference in performance, the much more massive basal lobes can be removed altogether without any significant effect upon tactile learning.

The inferior frontal system is subdivided into three anatomically distinct regions, one of which—the subfrontal lobe—includes more than nine-tenths of the nerve cells in the system. In the present series of experiments no animal was observed to learn after removal of the whole of the subfrontal lobe, though several were demonstrably capable of learning with only a fraction of it remaining. The size of the minimal fraction necessary was greater in discrimination experiments than in repeated presentation experiments (Table 4).

The observation that removal of the subfrontal lobe abolishes tactile learning does not, of course, itself show that organizational changes associated with touch learning are limited to this lobe. The subfrontal is enclosed within the median inferior frontal lobe (above) and the lateral inferior frontal lobes (at the sides—see Fig. 1); neither of these can be removed without cutting off a source of input from the arms to the subfrontal. The lateral inferior frontal lobes are themselves enclosed by tracts ascending to the median inferior frontal so that it is technically exceedingly difficult to remove them while leaving the source of input to the sub-

frontal through the median inferior frontal intact, and so far this has not been attempted. We can, however, show comparatively easily that animals remain capable of learning to reject objects in the absence of any input from the median inferior frontal lobe, and this implies that the median inferior frontal, at least, is not essential for the organizational changes presumed to accompany alteration of responses to an object touched.

This raises the question of median inferior frontal lobe function, and here the significant observation would appear to be that no transfer of effect of experience between arms occurs without it. Thus B 171 was trained using the second right arm, and this prolonged training did not affect the animal's subsequent performance with the second left arm; B 171 had an approximately symmetrical lesion including removal of the median inferior frontal lobe. The performance of this animal may be compared with that of D 47, where the median inferior frontal was intact. The second (LHS) arm of D 47 clearly benefited from the experience of the first (RHS) arm to be trained (Table 2). These results suggest that the median inferior frontal is important as a distributive region ensuring that information about what has happened to one arm is available to the others, a finding that is consistent with the anatomical observation that the median inferior frontal is a mass of interweaving tracts with only a relatively thin cortex of nerve cell bodies (cf. the subfrontal lobe where cell bodies form the greater part of the mass of the lobe). When experience is deliberately limited to one particular arm, as in most of the experiments quoted in this account, the presence or absence of the median inferior frontal becomes unimportant.

The present experiments do not allow us to distinguish between the functions of the subfrontal and lateral inferior frontal lobes, but it seems not unlikely on structural grounds that the latter is the final relay station for executive messages to the arms and not primarily concerned in any of the organizational changes associated with memory formation. The nerve cell bodies forming the walls of the lateral inferior frontal are, for the most part, comparatively large (nuclei 5–10 μ) and few (60,000 in the whole—both sides—of the lateral inferior frontal) with large axons running from them, and as such seem likely to prove motor in function in contrast to the very much more numerous ($5{\cdot}2 \times 10^6$) cells of the subfrontal which are nearly all very small (nuclei $< 5\,\mu$, densely packed with little cytoplasm) with small axons. It seems legitimate to guess from this that it is within the subfrontal that any organizational changes associated with changes in response to objects touched actually take place.

It is interesting that these changes in response can be determined by events occurring in a comparatively small number of cells. Some of the animals in the present series of experiments learned after removal of all but a few tens of thousands of cells from the inferior frontal system. B 126 (RHS), for example, with less than 39,000 cells left (of which 25,000 were subfrontal) ceased to accept the test object after three mistakes only (Table 4). As there is some functional evidence for subdivision of the tactile learning system into neurone fields related to the individual arms (Wells, 1959), the nerve cells actually concerned in the changes leading to

a reversal of response towards an object touched may well in a case such as this be numbered only in thousands or possibly even in hundreds.

The anatomy of the inferior frontal system may be compared with that of the optic-superior frontal-vertical lobe system shown by Boycott & Young (1950–7) to determine visual learning. Structurally there is a strong superficial resemblance between the median inferior frontal and the superior frontal, and between the subfrontal and vertical lobes. In both cases a 'distributive' region of tracts leads into a mass of very small cells and the parallel can be taken further by comparing the structural relations of the tactile lateral inferior frontal with those of the subvertical lobe in the visual system.

The functional parallel between the components of the tactile and visual systems does not agree closely with the structural, however. Octopuses can still be taught to recognize figures seen after removal of the superior frontal and vertical lobes (Boycott & Young, 1957), whereas removal of structurally similar parts from the tactile system abolishes touch learning altogether. Extensive damage to the tactile learning system does not appear to affect visual responses (Wells, unpublished), while removal of the vertical lobe certainly impairs touch learning. These differences could be interpreted as showing that the inferior frontal system is the functional equivalent of the optic lobes and not of the superior frontal-vertical lobe system. The structural evidence clearly argues against any such conclusion, however, and it seems more reasonable to believe that the optic lobes have additional properties not found in the axial nerve cords of the arms (where experience does *not* lead to changes in output). The apparent anomaly that while removal of the vertical lobe affects touch learning, removal of the subfrontal does not, so far as is known, impair visual learning may be explicable from the relative bulk of the parts concerned. The vertical lobe contains upwards of $2{\cdot}5 \times 10^7$ nerve cells, the subfrontal *c.* $5{\cdot}2 \times 10^6$ and one might well expect the magnitude of any effect of subfrontal removal on visual learning to be small compared with the effect of vertical lobe removal on touch; it is possible that careful quantitative study would reveal effects of subfrontal removal on visual learning that the (very few) experiments so far made have failed to detect.

That the tactile system is somewhat less elaborate than the visual is not surprising if octopuses are descended from pelagic ancestors as seems probable (see, for example, Roger, 1944; Yonge, 1947). It must be remembered that *Octopus* is far from being a typical cephalopod, or even a typical octopod and that, so far as we know, it is only within the Octopodidae themselves that the arms are used as a means of investigating the environment, recognizing and collecting food by touch—decapods use their arms only to grab things that they have (presumably) recognized by sight.* Associated with the specialized *Octopus*-like use of the arms for groping under rocks and into crannies after food is the development of a new extension of the inferior frontal system in the Octopodidae—the subfrontal lobe, which we now

* Practically nothing is known of the feeding habits of the vast majority of cephalopods and the views expressed in this discussion are necessarily based upon observation of the few species that can be kept in the laboratory, notably *Octopus* itself, *Sepia* and *Loligo*.

have reason to believe is a learning mechanism, adding a capacity to learn to recognize objects by touch to a capacity to remember things seen. In decapods there is no subfrontal lobe (Thore, 1939) and the inferior frontal is smaller in size compared with the components of the visual learning system (Wirz, 1954).

SUMMARY

1. Octopuses were trained to reject objects touched, after section of the optic nerves and removal of parts from the supraoesophageal brain mass.

2. Removal of the vertical lobe has effects, described in full elsewhere, that may be broadly described as reducing the efficiency of the learning process—the animals require more trials to attain the same standards of accuracy of response as controls.

3. Additional removal of the basal lobes produces no further decline in learning performance, although such animals show postural defects and cannot make movements that entail integration of the activities of the individual arms.

4. Extension of the lesion to remove the inferior frontal and subfrontal lobes produces animals that cannot be taught to change their reactions towards objects touched, although the movements concerned in taking or rejecting objects by single arms remain unimpaired. The latter are integrated within the axial cords of the arms concerned, and can be demonstrated in isolated arms.

5. Evidence is given that the organizational changes constituting learning are limited to the lateral inferior frontal and subfrontal lobes, and that the median inferior frontal is a distributive region. Animals with only a few tens of thousands of cells left in the lateral inferior frontal/subfrontal system can learn to reject an object repeatedly presented, but rather more tissue must remain before a satisfactory performance is obtained in discrimination experiments.

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EXPERIMENTS ON THE SELECTION OF ALGAL SUBSTRATES BY POLYZOAN LARVAE

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(With Plate 12)

INTRODUCTION

Polyzoa in the British Isles are found growing on a number of substrates: algae, wooden piles, stones, shells, hydroids, tunicates and other polyzoans. Although complete exclusiveness is rare, many species seem normally to prefer one sort of substrate. A number of species grow on algae, ranging from *Electra pilosa* (L.), an abundant species found on all types of substrate including many species of alga, to others such as *Membranipora membranacea* (L.) and *Amathia lendigera* (L.) which are found only on one or two algae.

The number of species of Polyzoa found in the intertidal zone is rather small, although some of them, such as *Flustrellidra hispida* (Fabricius), may be abundant. Most of these species grow as tufts or incrustations on the commoner algae, and the following associations are characteristic of British shores:

Crisia eburnea (L.): often quite abundant in the infra-littoral fringe (Stephenson & Stephenson, 1949) on small red algae, notably *Phyllophora membranifolia.* (The nomenclature of algae follows the list given by Parke (1953).)

Membranipora membranacea: typical of the infra-littoral fringe on the fronds of *Laminaria digitata* and *L. hyperborea*, occasionally on *L. saccharina*, *Saccorhiza polyschides* and *Fucus serratus.*

Celleporella hyalina (L.): on more exposed shores found in small amounts on a number of algae, especially in *Laminaria* holdfasts, but in certain sheltered localities it forms heavy incrustations on the fronds of *L. saccharina.*

Alcyonidium polyoum (Hassall), *A. hirsutum* (Fleming) and *Flustrellidra hispida*: all three species are found abundantly only on *Fucus serratus.*

Bowerbankia imbricata (Adams): an abundant species on *Ascophyllum nodosum.*

Amathia lendigera: abundant on *Halidrys siliquosa.*

Valkeria uva (L.): the small creeping variety (f. *uva*) grows on a number of substrates, but the luxuriant tufts of f. *cuscuta* have only been found on *Halidrys siliquosa.*

There has been no major study of the association between Polyzoa and algae in the British Isles comparable with that of Rogick & Croasdale (1949) for the Woods Hole region. However, some information is given by Hincks (1880), and the data of Joliet (1877), Prenant & Teissier (1924), Prenant (1927, 1932), and Bock (1950) are relevant.

From the examples given above it will be seen that all the Polyzoa, with the exception of *Bowerbankia imbricata*, are characteristically found on algae growing in

the lowest regions of the shore; moreover, when they are found principally on one alga it is usually the dominant species. This is true of *Flustrellidra* and *Alcyonidium* spp. which incrust the thallus of *F. serratus*, which, on a rocky shore, is usually the dominant species in the lowest part of the mid-littoral zone. Similarly, in sheltered areas of the Menai Straits where *Celleporella*, *Amathia* and *Valkeria* are common, the two dominant algae are *L. saccharina* and *Halidrys siliquosa*—the species on which they are found. The question is therefore posed as to whether the association of Polyzoa with algae is largely fortuitous—whether they occur most commonly on the dominant alga in that region of the shore which, because of a variety of other environmental factors, is most suited to their establishment, survival and growth—or whether the position on the shore is determined partly or largely by preferential settlement by the larvae on one particular algal species.

Polyzoan larvae belong to one or other of two basic types. In species like *Electra pilosa* and *Membranipora membranacea* there is a Cyphonautes larva with a planktotrophic pelagic phase of up to several weeks. The majority of species, however, have a lecithotrophic larva which settles shortly after liberation. The latter type was considered more suitable for experiments, and *Celleporella hyalina*, *Alcyonidium polyoum*, *A. hirsutum* and *Flustrellidra hispida*, all readily obtainable species, were investigated to discover the settlement preferences of the larvae.

METHODS

Experimental design

The first experiments were conducted with larvae of *Alcyonidium hirsutum*. As this species is found most commonly on *Fucus serratus*, this alga was used as a standard and, in each experiment, the larvae were offered a choice between it and one other species. This method, however, required a steady supply of large numbers of larvae, and was slow to produce results. Further, the most rigid precautions had to be taken to counteract any possible inequalities in other environmental factors such as lighting, which might have affected the choice of algae by influencing the behaviour of the larvae. Similar arguments apply to the 'American tournament' method used by Gross & Knight-Jones (1957), in which a number of pairs of algae were tested against each other in turn. Here again it is difficult to maintain sufficiently rigidly controlled conditions, while an unnecessarily large number of experiments have to be set up to yield a given amount of information.

A method was therefore required which did not suffer from these two disadvantages. The number of experiments could be reduced if it were possible to test a number of algae simultaneously, but, for reasons of economy of larvae and limitations of space, a complete block design large enough to include every alga in every possible combination was clearly impossible. On the other hand, an experiment in which each alga was included only once would be liable to considerable experimental error unless rigidly uniform environmental conditions could be achieved; consequently the design had to include some replicates. Valid and efficient comparisons of this kind can be made using incomplete randomized block designs.

The particular design chosen as most suitable for such experiments with marine larvae is shown in Table 1. The following notation has been employed:

A, B, C, ..., K = treatments (i.e. species of alga being offered for larval settlement).
t = number of treatments (i.e. total number of algal species offered).
$b\,[= tr/k = N/k]$ = number of blocks of treatments (i.e. total number of dishes used in the experiment).
k = number of treatments per block (i.e. pieces of alga in each dish).
r = number of replications of each treatment (i.e. number of times each algal species is offered with others in the experiment).
$N\,[= tr]$ = total number of observations (i.e. total number of pieces of alga of all species used in the experiment).
$\lambda\,[= r(k-1)/(t-1)]$ = number of blocks (dishes) in which any two given treatments (algae) occur together.
I, II, ..., V = positions in the block. These positions were allocated in relation to the orientation of the dish to the laboratory bench.

Table 1. *Symmetrically balanced incomplete randomized block design used for settlement experiments. Eleven algae can be compared using eleven dishes with five pieces of alga in each. The five positions in the dishes are indicated by Roman numerals*

Algae	Dishes										
	1	2	3	4	5	6	7	8	9	10	11
A	I	—	—	V	—	IV	III	II	—	—	—
B	—	I	—	—	V	—	IV	III	II	—	—
C	—	—	I	—	—	V	—	IV	III	II	—
D	—	—	—	I	—	—	V	—	IV	III	II
E	II	—	—	—	I	—	—	V	—	IV	III
F	III	II	—	—	—	I	—	—	V	—	IV
G	IV	III	II	—	—	—	I	—	—	V	—
H	—	IV	III	II	—	—	—	I	—	—	V
I	V	—	IV	III	II	—	—	—	I	—	—
J	—	V	—	IV	III	II	—	—	—	I	—
K	—	—	V	—	IV	III	II	—	—	—	I

The design shown in Table 1 is more than just an incomplete randomized block design: it is a Youden Square (Davies, 1954). A Youden Square has the following important characteristics:

(i) The design is balanced in respect of treatments, any given pair occurring together the same number of times as any other pair. This is clearly important in choice experiments where all comparisons between any two algae are equally important. This balancing allows the calculation of treatment effects corrected for any differences between blocks.

(ii) The design is balanced with respect to blocks as well as treatments. It is then said to be symmetrical and the necessary condition is that $b = t$, and con-

sequently $k = r$. Only if the design is symmetrical is it possible to calculate block effects corrected for differences between treatments.

(iii) Any symmetrically balanced incomplete block design will become a Youden Square provided that each treatment occurs once, and once only, in each position. This is particularly important since treatment and block effects are then independent of any position effects.

The use of an experimental design which is a Youden Square therefore brings a number of advantages, notably that the effects due to treatments, blocks and positions can be evaluated separately. In settlement experiments it is especially important that the effects due to the algae are not influenced by variations due to position, such as might be caused through uneven illumination. The number of designs fulfilling these conditions is very limited, but suitable ones exist for testing seven or eleven treatments simultaneously. In the design chosen eleven algae could be tested in eleven blocks (glass dishes), with five positions in each dish. Further particulars of incomplete randomized block designs are given by Davies (1954).

General experimental technique

Weed bearing breeding polyzoan colonies was collected at low tide, or else by grappling from a dinghy. *Celleporella* and *Flustrellidra* are summer breeders, while the *Alcyonidium* spp. breed in the autumn. The collected material was kept in large tanks, and was found not to deteriorate over a period of several days; but the larvae were never used after the adult colonies had been in aquaria for more than 3 days. The tanks were kept in the dark, with good aeration and a generous flow of sea water. In the mornings the dark covers were removed and the water circulation stopped. Within a couple of hours hundreds of larvae had been liberated in response to light, and these were gently pipetted out into experimental dishes.

The algae used in the choice experiments were collected from rather exposed shores. In this way clean material could be obtained which was not already colonized by polyzoans. Although settled larvae of *Celleporella* and *A. hirsutum* did not appear to have any influence on further settlement, it seemed advisable, because of gregarious tendencies in some other settling larvae, to use perfectly clean weed. Freshly collected non-fruiting fronds only were used in the experiments.

Each experiment was set up as follows: eleven glass finger-bowls, 12 cm. in diameter, were scrupulously cleaned and rinsed, and placed on the wet bench of a basement aquarium. In this room the temperature during the summer remains in the range of 14·5–17·5° C., and is virtually constant throughout the day. Light was provided mainly by a fluorescent strip directly over the dishes.

It is important that variables under the experimenter's control should be randomized. Consequently, using tables of random numbers, the dishes were numbered, the positions decided, and the algae to be tested were allocated letters. Pieces of thallus were cut from the algae, each being of comparable area to the tip of a *Fucus serratus* frond about 2 cm. long. In the case of branching species like *Chondrus* and *Furcellaria* this could not be very accurately judged, but the effect of variations in area was minimized by the use of a number of replicates. Moreover,

with a small number of larvae per dish and ample settlement space on all the pieces of weed offered, it is highly unlikely that even considerable inequalities in size would add significantly to the inevitable random variation due to small numbers settling. The pieces of alga were cut the evening before the experiment and the amount of cut edge was kept to a minimum; they were then thoroughly washed the following morning. These precautions were taken to reduce exudation of mucus in which the larvae might be trapped.

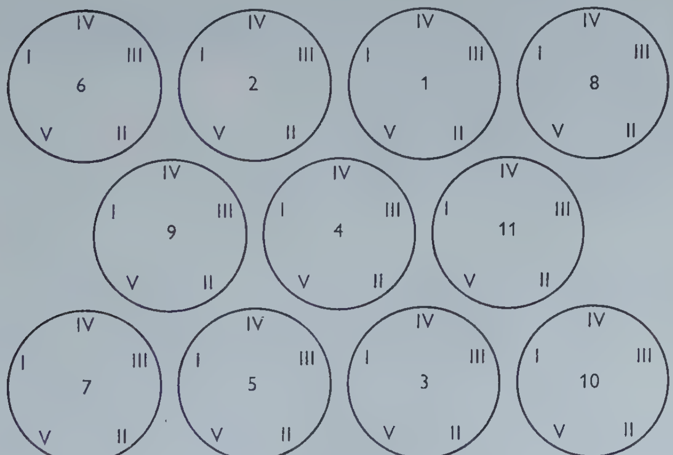
The pieces of alga were placed in the finger bowls of sea water, according to the pattern indicated by Table 1, and roughly positioned. Twenty-five larvae were gently placed in each dish using a moderately wide-mouthed pipette, and the algae finally positioned with a glass rod. After preparing all the dishes the experiment was left undisturbed for 24 hr.; then the settlement on each piece of alga was recorded. An example of the method of analysis of the results is given below.

Analysis of results

In one experiment using twenty-five larvae of *A. hirsutum* per dish, the algae were randomly designated the following letters:

A	*Fucus spiralis*	F	*Chondrus crispus*
B	*Furcellaria fastigiata*	G	*Ulva lactuca*
C	*Pelvetia canaliculata*	H	*Laminaria digitata*
D	*Polyides caprinus*	I	*Fucus serratus*
E	*Fucus vesiculosus*	J	*Gigartina stellata*

K *Ascophyllum nodosum*



Text-fig. 1. Layout of algal choice experiment in accordance with a Youden Square design (see text).

The dishes were arranged on the bench and randomly numbered. The algae were then placed in them in accordance with Text-fig. 1, Table 1 and the letters designated above. The results are recorded in Table 2, which shows the manner in which all the data were entered.

Table 2. *Settlement recorded on eleven algal substrates in a choice experiment in which twenty-five* Alcyonidium hirsutum *larvae were added to each dish*

Dishes	Positions					Dish totals (B)
	I	II	III	IV	V	
1	*F. spiralis* 4	*F. vesiculosus* 6	*Chondrus* 1	*Ulva* 0	*F. serratus* 6	17
2	*Furcellaria* 0	*Chondrus* 0	*Ulva* 0	*L. digitata* 0	*Gigartina* 6	6
3	*Pelvetia* 3	*Ulva* 0	*L. digitata* 0	*F. serratus* 13	*Ascophyllum* 0	16
4	*Polyides* 0	*L. digitata* 0	*F. serratus* 7	*Gigartina* 5	*F. spiralis* 7	19
5	*F. vesiculosus* 0	*F. serratus* 20	*Gigartina* 4	*Ascophyllum* 0	*Furcellaria* 0	24
6	*Chondrus* 5	*Gigartina* 7	*Ascophyllum* 2	*F. spiralis* 8	*Pelvetia* 0	22
7	*Ulva* 0	*Ascophyllum* 0	*F. spiralis* 13	*Furcellaria* 1	*Polyides* 0	14
8	*L. digitata* 0	*F. spiralis* 9	*Furcellaria* 0	*Pelvetia* 2	*F. vesiculosus* 0	11
9	*F. serratus* 14	*Furcellaria* 0	*Pelvetia* 8	*Polyides* 0	*Chondrus* 3	25
10	*Gigartina* 4	*Pelvetia* 4	*Polyides* 0	*F. vesiculosus* 0	*Ulva* 0	8
11	*Ascophyllum* 0	*Polyides* 0	*F. vesiculosus* 9	*Chondrus* 1	*L. digitata* 0	10
Position totals (L)	30	46	44	30	22	172

The settlement figures were later re-tabulated in the form shown in Table 3, and the corrected values for the means calculated. The numerical values were as follows:

t (the number of algae being tested) $= 11$,
k (the number of algae per dish) $= 5$,
r (the number of replicates) $(= k) = 5$,
N (the total number of observations) $= 55$,
b (the number of dishes) $(= t) = 11$.

Also:

$G =$ the total number of larvae settled,
$T_A, T_B, \ldots, T_K =$ number of larvae settled on the algae A, B, ..., K,
$B_1, B_2, \ldots, B_{11} =$ number of larvae settled in the dishes 1, 2, ..., 11,
$L_I, L_{II}, \ldots, L_V =$ number of larvae settled in the positions I, II, ..., V.

As any one of the algae, A, B, ..., K, is present in only r of the b dishes, the settlement totals, T_A, T_B, etc., are influenced by random differences in the dishes and are not entirely indicative of true differences in favourability of the algae to the larvae under consideration. But analysis can separate block (dish) and treatment (algal) effects. Since the design is balanced and symmetrical it is possible to estimate the treatment effects free from block effects, and vice versa (Davies, 1954):

(i) For each alga calculate the value of Q_A, Q_B, etc., where $Q_A = kT_A -$(sum of all the totals of the dishes containing the alga A).

Table 3. *The results shown in Table 2 for the settlement of* Alcyonidium hirsutum *larvae on eleven algal substrates re-tabulated for analysis (see text)*

Algae	Dishes 1	2	3	4	5	6	7	8	9	10	11	Totals (T)	Q	P	Corrected treatment means
A (*F. spiralis*)	4	—	—	7	—	8	13	9	—	—	—	41	122	5·54	8·69
B (*Furcellaria*)	—	0	—	—	0	—	1	0	0	—	—	1	−75	−3·41	−0·26
C (*Pelvetia*)	—	—	3	—	—	0	—	2	8	4	—	17	3	0·14	3·29
D (*Polyides*)	—	—	—	0	—	—	0	—	0	0	0	0	−76	−3·46	−0·31
E (*F. vesiculosus*)	6	—	—	—	0	—	—	0	—	0	9	15	5	0·23	3·38
F (*Chondrus*)	1	0	—	—	—	5	—	—	3	—	1	10	−30	−1·36	1·79
G (*Ulva*)	0	0	0	—	—	—	0	—	—	0	—	0	−61	−2·77	0·38
H (*L. digitata*)	—	0	0	0	—	—	—	0	—	—	0	0	−62	−2·82	0·33
I (*F. serratus*)	6	—	13	7	20	—	—	—	14	—	—	60	199	9·05	12·20
J (*Gigartina*)	—	6	—	5	4	7	—	—	—	4	—	26	51	2·32	5·47
K (*Ascophyllum*)	—	—	0	—	0	2	0	—	—	—	0	2	−76	−3·46	−0·31
Totals (B)	17	6	16	19	24	22	14	11	25	8	10	G 172	0	0	—
													G/N		
Q'	−41	−7	1	−32	16	14	26	−19	37	−18	23	0	3·15	—	—
P'	−1·86	−0·32	0·04	−1·45	0·73	0·64	1·18	−0·86	1·68	−0·82	1·04	0	—	—	—
Corrected block means	1·29	2·83	3·19	1·70	3·88	3·79	4·33	2·29	4·83	2·33	4·19	—	—	—	—

(ii) Obtain the value of P_A, P_B, etc. by dividing the appropriate Q value by $N(k-1)/(t-1)$. The sums of both the Q's and P's should be zero. The corrected treatment means—estimates of the settlement that would have taken place if all the algae were present in every dish—are obtained by adding P_A, P_B, ..., P_K in turn to the grand mean G/N.

The block effects can be similarly calculated:

(iii) $Q'_1 = rB_1-$(sum of the totals of all the treatments occurring in dish 1): and so on.

(iv) Obtain the value of P'_1 by dividing Q'_1 by $N(r-1)/(b-1)$: and so on. Since in this design $r = k$ and $b = t$ the divisors for obtaining P's (ii) and P''s (iv) are identical.

In comparing the favourability of the different algae it is the corrected treatment means that are used (Text-figs. 2–5), but the P's and P''s are required for the analysis of variance. These values are shown in Table 3.

The analysis of variance is based on the assumption that the number of larvae settled on any given piece of alga can be represented by

$$Y_{ijk} = M+T_i+B_j+L_k+e_{ijk},$$

where Y_{ijk}, the number settling on the alga i in position k in dish j, will be a constant M, plus a factor T_i depending on the quality of the alga, plus a dish factor B_j, plus a position factor L_k, plus a random (error) term e_{ijk} (Davies, 1959). The main purpose of the analysis of variance is to obtain an estimate of the error e_{ijk}. Using this estimate the magnitude and significance of each T_i, each B_j and each L_k can be assessed. The analysis of variance table provides a convenient way of assessing whether the B_j's or L_k's as a whole add significantly to the variation. The analysis of variance—summarized in Table 4—using the data from Tables 2 and 3 is conducted as follows (Davies, 1954):

(v) Find the correction due to the mean $= G^2/N = 172^2/55 = 537{\cdot}9$. This will be used in later calculations.

(vi) Calculate the total sum of squares of all N observations about the grand mean, i.e. $\Sigma x^2 - G^2/N = 1622-537{\cdot}9 = 1084{\cdot}1$.

(vii) Calculate the sum of squares due to the corrected treatment totals, i.e. $\Sigma Q_A{}^2$, $Q_B{}^2$, etc. divided by $Nk(k-1)/(t-1) = 82762/110 = 752{\cdot}4$.

(viii) Calculate the sum of squares due to the actual block totals. This is the sum of squares of B_1, B_2, etc. divided by k, minus the correction due to the mean, i.e. $\Sigma B^2/k - G^2/N = 3108/5-537{\cdot}9 = 83{\cdot}7$.

(ix) Calculate the sum of squares due to the corrected block totals, i.e. $\Sigma Q_1'^2$, $Q_2'^2$, etc. divided by $Nr(r-1)/(b-1) = 6466/110 = 58{\cdot}8$. (Since $r = k$ and $b = t$, the divisors in (vii) and (ix) are identical.)

(x) Calculate the sum of squares due to the actual treatment totals. This is the sum of squares of T_A, T_B, etc. divided by r, minus the correction due to the mean, i.e. $\Sigma T^2/r - G^2/N = 6576/5-537{\cdot}9 = 777{\cdot}3$.

(xi) Calculate the sum of squares due to the position totals. This is the sum of

squares of the position totals $L_{\mathrm{I}}, \ldots, L_{\mathrm{V}}$ divided by b, minus the correction due to the mean, i.e. $\Sigma L^2/b - G^2/N = 6336/11 - 537.9 = 37.9$.

Table 4. *Analysis of variance on the data given in Tables 2 and 3 (also see text)*

Source of variation	Sum of squares		Degrees of freedom		Mean square
Tests for treatments (algae):					
(vii) between algae, adjusting for blocks (dishes)	$\frac{(t-1)}{Nk(k-1)}\Sigma Q^2$	752·4	$t-1$	10	75·24
(viii) between dishes, ignoring treatments (algae)	$(\Sigma B^2/k - G^2/N)$	83·7	$b-1$	10	(Discard)
	Total	836·1		20	
Tests for blocks (dishes):					
(ix) between dishes, adjusting for treatments (algae)	$\frac{(b-1)}{Nr(r-1)}\Sigma Q'^2$	58·8	$b-1$	10	5·88
(x) between algae, ignoring blocks (dishes)	$(\Sigma T^2/r - G^2/N)$	777·3	$t-1$	10	(Discard)
	Total	836·1		20	
(xi) between positions	$(\Sigma L^2/b - G^2/N)$	37·9	$k-1$	4	9·475
Remainder = interaction between treatments, blocks and positions = error variance, s^2	Total − [(vii) + (viii) + (xi)] or Total − [(ix) + (x) + (xi)]	210·1	$N-t-b-k+2$	30	7·00
Total	$\Sigma x^2 - G^2/N$	1084·1	$N-1$	54	

These data may then be presented as in Table 4. Totals (vii)+(viii) = (ix)+(x), and this common figure+(xi) subtracted from the total sum of squares (vi) gives the remainder or error variance. The mean squares are then found by dividing the sums of squares by the appropriate number of degrees of freedom. However, the mean squares of (viii) and (x), as shown in Table 4, are discarded since these have been derived on the assumption that there are no treatment differences in (viii) or block differences in (x). The sums of squares were required to arrive at the error variance, but the mean squares themselves would have no value.

An indication of significance is obtained by comparing the ratio of the treatment (or block, or position) mean square to the error mean square with the appropriate F-value. Thus the ratios of the mean squares are:

(*a*) Treatment: 10·75. The F-value for 10 and 30 degrees of freedom is 2·16 at the 5 % level. The treatment value of 10·75 is clearly highly significant.

(*b*) Block: 0·84. Obviously the block effects are not significant.

(*c*) Position: 1·35. The F-value for 4 and 30 degrees of freedom is 2·69 at the 5 % level. The position value of 1·35 is not significant.

The standard deviation of any two of the corrected treatment means is

$$s\sqrt{\left\{\frac{2k(t-1)}{N(k-1)}\right\}},$$

where s^2 is the error mean square. Using the above figures

$$\sqrt{\frac{7\cdot0 \times 100}{220}} = \sqrt{3\cdot18} = 1\cdot78.$$

The difference required for significance between treatment means is

$$1\cdot78 \times 2\cdot04 = 3\cdot63,$$

where 2·04 is the 5 % value of t for 30 degrees of freedom. It can be seen that some of the differences between the corrected treatment means (Table 3) are highly significant.

RESULTS AND DISCUSSION OF CHOICE EXPERIMENTS

Four species of Polyzoa have been tested by offering larvae a choice between different species of alga. In each case two series of experiments have been conducted using a slightly different range of algae in each series. In this way the larvae of each polyzoan have been offered a choice of fourteen or fifteen different algae on which to settle. The results—the corrected algal means—converted to percentage figures to allow comparison between experiments are given in Table 5. The figures for series II of *Alcyonidium hirsutum* were not obtained by the method previously described, but are based on a number of separate comparisons between *Fucus serratus* and any one other species. These results are also expressed as percentages and may thus be compared with those of series I.

The variance ratios for treatment, block and position effects are also shown in Table 5, those values significant at the 5 % probability level being marked with an asterisk. When, as in this case, a number of independent tests of significance have been made, it is possible to obtain a single test of the significance of the aggregate. The method (Fisher, 1950, p. 99) is based on the product of the probabilities of the individual values. By taking the natural logarithm of the probability, doubling it and changing the sign, the equivalent value of χ^2 for 2 degrees of freedom is obtained. Any number m of such values of χ^2 may be added together and tested for significance on the basis of $2m$ degrees of freedom.

This method has been applied in Table 6 to the variance ratios shown in Table 5. From Table 5 it will be seen that the treatment effects are generally highly significant, but that one of the values for *C. hyalina* is not so. But the combined probabilities give a χ^2 value of 74·0 for 8 degrees of freedom. For this species, therefore, as in the others, the overall treatment effect is extremely significant (Table 6).

The low F and χ^2 values due to block effects show that the experiments have not been influenced by differences in the blocks themselves. Position effects, however, are more important. The χ^2 values of the aggregates in *A. hirsutum*, *A. polyoum* and *F. hispida*, while fairly high, are not in fact significant; that for *C. hyalina* on the other hand shows a high degree of significance. This means that another factor—presumably light—has affected the settlement behaviour of these larvae. It is certainly true that larvae of this species swim more actively than those of the *Alcyonidium* spp. and *F. hispida*, and their behaviour is more conspicuously

Table 5. *Results of settlement experiments in which polyzoan larvae were offered a choice of algal substrates. The figures shown are the corrected means (see text) expressed as percentages. The variance ratios that are significant at the 5% probability level are marked with an asterisk*

Alga	*Alcyonidium hirsutum*				*Alcyonidium polyoum*				*Flustrellidra hispida*				*Celleporella hyalina*			
	IA	IB	IC	II	IA	IB	IIA	IIB	IA	IB	IIA	IIB	IA	IB	IIA	IIB
Ascophyllum nodosum	−0·2	−0·6	−0·9	3·3	0·3	1·5	—	—	0·7	4·0	—	—	3·0	1·0	—	—
Chondrus crispus	22·0	9·3	4·9	14·0	8·4	9·3	0·3	3·4	2·4	2·7	8·3	−1·5	29·2	20·3	24·4	11·7
Dictyota dichotoma	—	—	—	—	—	—	25·1	7·7	—	—	20·6	19·6	—	—	3·9	7·9
Fucus serratus (fruiting tips)	—	—	—	12·2	—	—	—	—	—	—	—	—	—	—	—	—
F. serratus (non-fruiting tips)	36·3	37·5	33·6	35·8	35·8	30·4	29·6	38·7	44·5	30·5	24·0	19·7	16·0	2·8	21·2	19·1
F. spiralis	12·0	21·8	23·9	—	9·7	15·3	—	—	21·4	25·2	—	—	10·0	1·7	14·1	8·2
F. vesiculosus	3·0	3·9	9·3	9·2	8·8	9·3	23·5	32·3	10·7	18·8	13·8	30·8	−0·3	0·8	7·0	15·0
Furcellaria fastigiata	6·2	0·1	−0·7	—	3·7	1·8	3·2	4·1	1·2	1·4	2·5	1·4	−1·2	4·5	—	—
Gigartina stellata	8·1	19·0	15·0	13·2	18·8	19·0	12·1	11·8	8·2	11·9	12·8	17·1	13·5	13·3	11·3	13·7
Halidrys siliquosa	—	—	—	—	10·0	6·1	—	—	—	—	—	—	—	—	−1·6	−1·8
Laminaria digitata	3·0	−0·7	0·9	—	—	—	−0·2	−0·3	0·4	5·4	−0·3	0·2	−1·0	5·4	2·4	4·1
L. hyperborea	—	—	—	—	0·1	1·4	—	—	—	—	—	—	—	—	—	—
L. saccharina	—	—	—	—	—	—	0·1	0·5	—	—	4·6	0·2	5·7	26·2	9·2	7·1
Pelvetia canaliculata	9·2	3·9	9·1	—	0·9	0·9	1·1	0	−0·5	1·1	2·8	0·8	17·7	21·0	2·1	6·3
Polyides caprinus	−0·1	2·2	−0·9	—	3·6	4·9	4·9	4·4	2·4	0·9	9·9	13·6	—	—	—	—
Rhodymenia palmata	—	—	—	1·8	—	—	—	—	8·5	−1·9	—	—	8·0	3·4	—	—
Ulva lactuca	−0·3	1·2	1·1	—	—	—	0·1	−1·6	—	—	1·5	−1·8	—	—	6·3	8·9
Amount by which means must differ to be significant at 5% probability level	14·6	10·2	9·9	—	8·2	10·2	9·9	8·3	15·5	12·5	12·2	10·9	8·0	10·4	12·0	12·2
Variance ratios:																
treatment : remainder (5% *F*-value = 2·16)	4·91*	12·0*	10·75*	—	13·38*	6·85*	11·20*	23·0*	6·31*	6·51*	3·52*	8·91*	11·58*	6·50*	4·08*	1·81
block : remainder (5% *F*-value = 2·16)	0·42	1·20	0·84	—	0·59	0·68	0·45	1·22	0·52	1·27	0·58	1·03	1·82	0·36	0·35	0·28
position : remainder (5% *F*-value = 2·69)	0·53	4·83*	1·35	—	1·94	1·13	1·21	1·10	0·38	0·71	0·42	3·00*	5·00*	2·69*	4·08*	3·26*
No. of larvae used in experiment	275	275	275	1761	275	275	275	275	275	275	220	110	550	275	275	275
Actual no. of larvae which settled	193	195	172	1207	189	175	179	208	81	103	87	71	374	211	243	218

influenced by light. This result also serves to stress that comparisons of treatments must be carried out in such a way as to be independent of possible positional effects.

The broad trend of the results can best be seen in Text-figs. 2–5, which show the average settlement values for each of the two series for the polyzoans investigated. These histograms show two general patterns. In *Flustrellidra* and the species of *Alcyonidium* there is a sharp falling away from one very favourable species, but in *Celleporella* several species are moderately favourable and the falling off is much more gradual.

In *Alcyonidium hirsutum*, *A. polyoum* and *Flustrellidra hispida*, the three truly littoral species investigated, settlement was highest on *Fucus serratus*—about 35 % (Text-figs. 2–4). All three polyzoans are, in fact, normally found on *F. serratus*. Text-fig. 2 shows that *A. hirsutum* larvae greatly preferred non-fruiting tips to fertile ones, and this is no doubt true for the other species. Probably this is due to the exudation of mucus from the conceptacles.

For *A. hirsutum* three other algae were moderately favourable for settlement: *F. spiralis*, *Gigartina stellata* and *Chondrus crispus*. *Dictyota* was not tested. There is little doubt that *F. spiralis* occurs too high on the shore for the survival of *A. hirsutum*: possibly desiccation would be too great, or the time available for feeding too short. On sheltered shores, such as those in the Menai Straits, where *Chondrus* and *Gigartina* are scarce, *A. hirsutum* is found almost exclusively on *F. serratus*; but on more exposed coasts where red algae are numerous small colonies are found abundantly on *Gigartina* and *Chondrus* as well as on *F. serratus*.

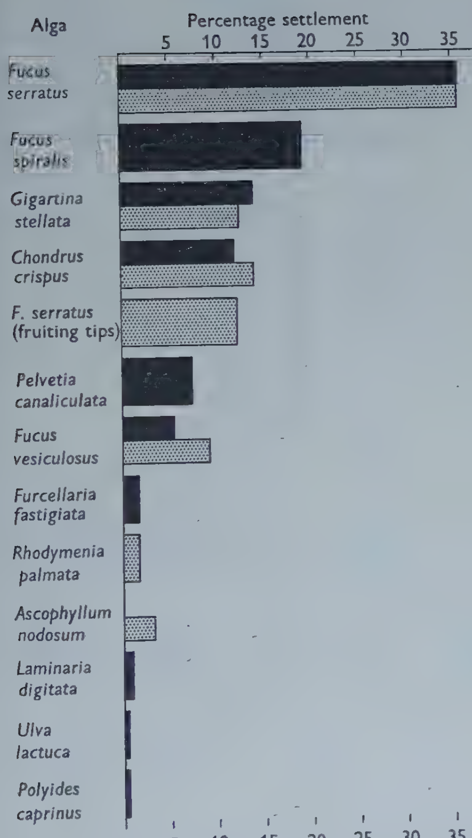
The position is similar with regard to *A. polyoum* (Text-fig. 3). In series II *F. vesiculosus* proved very favourable for settlement, but was much less so in series I. Although *F. vesiculosus* occurs lower than *F. spiralis*, its greatest abundance being in the middle of the intertidal zone, this level may still be too high for successful colonization by the larvae. *Gigartina* also was fairly favourable for settlement, a fact which accords well with observations on more exposed shores. Gross & Knight-Jones (1957) found that *Dictyota* was favourable for settlement by *Spirorbis borealis* larvae, and the same holds true for the polyzoan larvae investigated. But *Dictyota* is never sufficiently abundant to provide an important substrate for polyzoan zoaria; it is primarily an infra-littoral form, although sometimes occurring in pools on the shore. Also, the fronds are very shortlived.

Larvae of *Flustrellidra hispida* show similar preferences to those of *A. polyoum* (Text-fig. 4). Similar reasons may be offered for its absence under natural conditions from the apparently favourable algae *F. spiralis*, *F. vesiculosus* and *Dictyota*. As with the species of *Alcyonidium*, on sheltered shores *Flustrellidra* is virtually confined to *F. serratus*, but where exposure is greater and *Gigartina* is present zoaria may be found on both species.

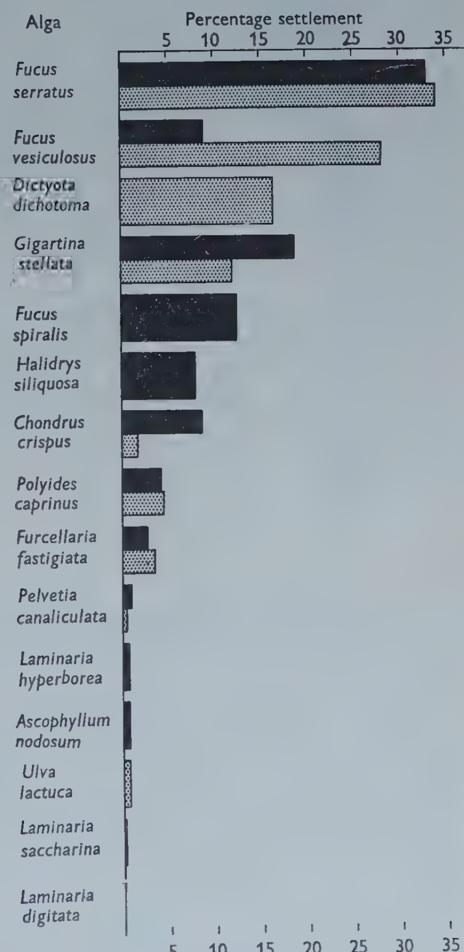
In these ctenostomatous forms, which characteristically occur at the lower levels of the mid-littoral zone, it seems highly significant that *F. serratus* was shown by the experiments to be the most preferred species, and that *Gigartina* was also favourable. *Laminaria* spp. and other red algae received low settlement. The results show that positive selection by the larvae could play an important role in

Table 6. *Tests for the significance of effects due to treatment, block and position in data derived from experiments on the choice of algal substrates by polyzoan larvae. The values shown are those of χ^2 based on the aggregate probabilities of the variance ratios shown in Table 5. Values of χ^2 significant at the 5% probability level are marked with an asterisk*

Effect	Polyzoan			
	A. hirsutum (6 D.F.) χ^2	*A. polyoum* (8 D.F.) χ^2	*F. hispida* (8 D.F.) χ^2	*C. hyalina* (8 D.F.) χ^2
Treatment	79·4*	136·3*	79·0*	74·0*
Block	3·26	3·40	4·78	12·90
Position	9·60	9·35	8·60	34·2*

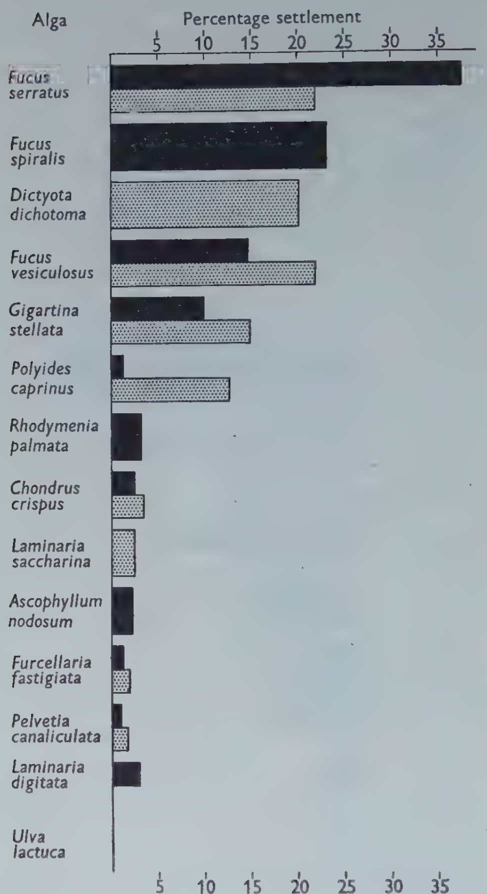


Text-fig. 2. The settlement of *Alcyonidium hirsutum* larvae on algae. Results are shown for two series of experiments.

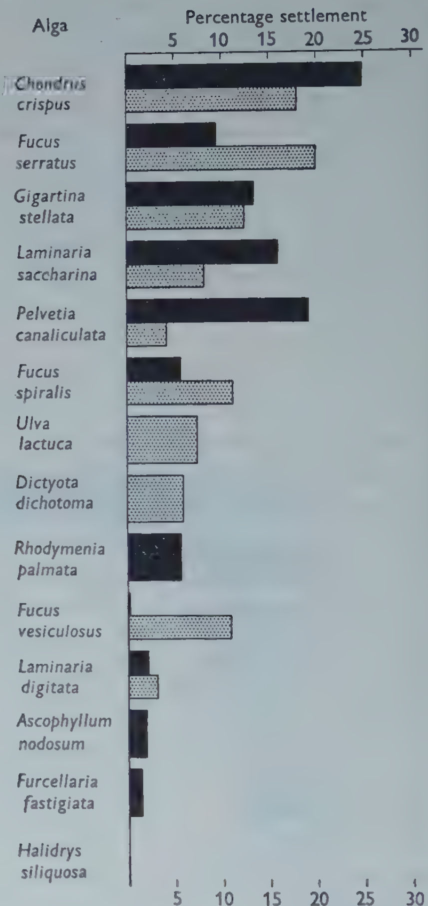


Text-fig. 3. The settlement of *Alcyonidium polyoum* larvae on algae. Results are shown for two series of experiments.

determining the distribution of the polyzoans on the shore, although the moderate favourability of *F. vesiculosus* and *F. spiralis* indicates that other factors must also operate.



Text-fig. 4. The settlement of *Flustrellidra hispida* larvae on algae. Results are shown for two series of experiments.



Text-fig. 5. The settlement of *Celleporella hyalina* larvae on algae. Results are shown for two series of experiments.

In the case of *Celleporella hyalina* the position is less clear (Text-fig. 5). The results do not show such clearly defined preferences as was the case with the other species. Settlement on the most favourable species was only about 20%, and several other algae were nearly as suitable. On rocky shores with moderate exposure *Celleporella* is found in small amounts on a number of algae, and most zoaria are found on the rhizoids of *Laminaria digitata* and *L. hyperborea*: it is not especially characteristic of *Chondrus crispus*. Possibly this preference is explicable in terms of shelter from wave exposure rather than in those of a most suitable surface.

In certain sheltered conditions, however, the growth of *Celleporella* is quite different. Where *L. saccharina* grows in abundance, then the polyzoan forms

extensive encrustations over the thallus. While the choice experiments did not show that *Celleporella* selected *L. saccharina* in preference to others, it was, nevertheless, a favourable species. This forms a significant contrast with *Flustrellidra* and the *Alcyonidium* spp. In the Menai Straits the locally abundant *L. saccharina* is found with *Halidrys* at just below low-water mark of ordinary spring tides. No larvae settled on *Halidrys*. Inasmuch as under these conditions the *Celleporella* is found on the *Laminaria*, the experiments again indicate that the larvae are making a definite choice. On more exposed shores, where many more species of alga are present, it seems that the distribution of *Celleporella* cannot be explained by this mechanism.

FACTORS INFLUENCING CHOICE OF ALGAL SUBSTRATE

Some experiments have been carried out to investigate certain of the factors thought to influence settlement on algal substrates.

Surface contour

Pelvetia and *Gigartina*, two of the algae used in the choice experiments, have fronds that are channelled. Settlement on the two surfaces of these species was recorded separately (Table 7). With the sole exception of the settlement of *A. hirsutum* on *Pelvetia*, the difference between the numbers of larvae settling on the two surfaces was very striking. The concave surface is obviously greatly preferred.

Table 7. *The influence of surfacc contour on settlement: differential settlement by polyzoan larvae on the two surfaces of algae having channelled fronds*

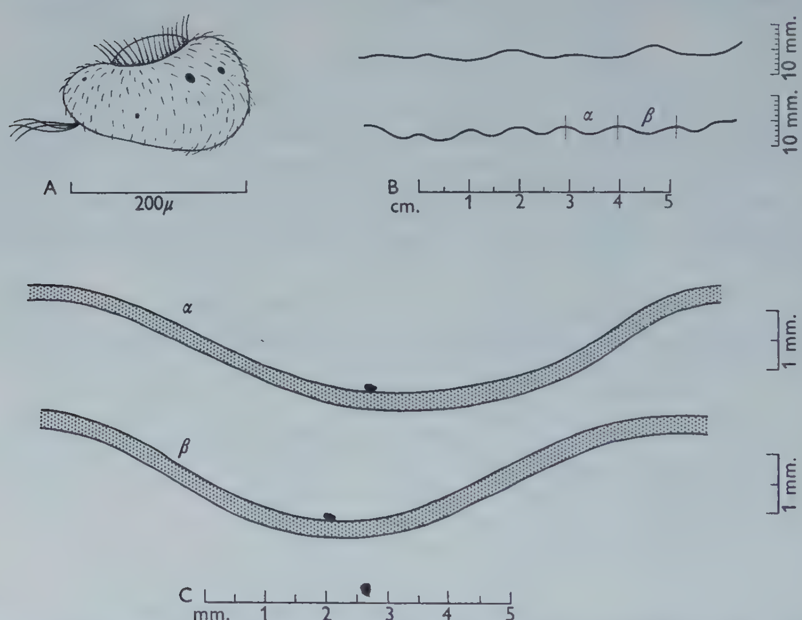
Alga	*Pelvetia*		*Gigartina*	
Polyzoan	Convex	Concave	Convex	Concave
Alcyonidium hirsutum	24	19	6	72
A. polyoum	1	6	7	101
Flustrellidra hispida	0	7	3	41
Celleporella hyalina	17	103	13	124

The frond of *Laminaria saccharina* is characteristically crinkled (Pl. 12*A*), and it is very noticeable that the young colonies of *Celleporella* are located in the concavities (Pl. 12*B*). In fact, if the *Laminaria* frond is held up to the light, it can be seen that the pattern of settlement on one surface is exactly complementary to that on the other, in each case closely following the surface contour. An experiment was conducted to discover whether the selection of concavities was a response to water movement or merely to surface contour. Transverse strips of *L. saccharina* frond were placed flat on the bottom of glass finger-bowls and *Celleporella* larvae gently pipetted into the dish. After 24 hr. the number of larvae that had settled in concavities, on convexities or on the flat marginal 'wing' area was counted. The results are shown in Table 8 and it can be seen that the density of set larvae was lowest on the convexities, intermediate, but still low, on the flat wing, and highest in the concavities. It is important to note that settlement on the upper (74) and

lower (80) surfaces of the frond was roughly equal. Clearly gravity cannot be the causal factor in the selection of concavities by the larvae: the behaviour would seem to be a direct response to the shape of the surface.

Table 8. *The influence of surface contour on settlement: selection by* Celleporella hyalina *larvae of concavities of* Laminaria saccharina *frond*

Part of frond	Area (cm.^{2})	No. set	Density (no. per cm.^{2})
Centre area (concavities)	35	87	2·49
Flat wing area	108	60	0·56
Centre area (convexities)	35	7	0·20



Text-fig. 6. Surface contour and larval settlement. (*A*) Larva of *Celleporella hyalina.* (*B*) Longitudinal profiles of the crinkled area of the two pieces of *Laminaria saccharina* used in the experiments on which Table 8 is based. (*C*) Enlarged sections through two concavities of the *L. saccharina* frond shown in (*B*), with *Celleporella* larvae indicated to scale.

The larva of *Celleporella*, illustrated in Text-fig. 6*A*, is dorso-ventrally flattened and covered with cilia. The length is 200–250 μ. It has four pairs of orange pigment spots which may assist in orientation to light, although they are not present in all polyzoan larvae. Before settling, the larvae glide over the surface, at the same time rotating about the vertical axis. In this way they can explore the substrate to find a suitable place for metamorphosis. (Further particulars of larval structure and metamorphosis are given by Barrois (1877) and Calvet (1900).) Fig. 6*B* shows a profile through the crinkled part of the *L. saccharina* fronds used in the experiment

summarized in Table 8. The size of the larva is indicated relative to that of the concavities in Fig. 6*C*: it seems remarkable that in perfectly still water the larva can detect the relatively slight curvature of the surface.

While the mechanism of site selection is not clear, definite advantages would seem to be obtained by settlement in concavities or in the channelled surface of a frond. The newly settled larva would receive protection from rubbing against rocks and other algae and from abrasion by sand. The latter factor may be important in the case of *Celleporella*, for *L. saccharina* may be found attached to stones on an otherwise sandy shore. Similar behaviour has been recorded during the settlement of other invertebrate larvae, such as barnacle cyprids (Crisp & Barnes, 1954) where it was also shown to be a direct tactile response to the shape of the surface.

It was previously suggested (Ryland, 1959) that in choice experiments settlement on *L. saccharina* would be higher if crinkled rather than flat pieces of frond were used. Later experiments have not supported this view: they suggest rather that contour is of subsidiary importance to surface texture. It seems probable that not all pieces of thallus of any given alga are equally favourable for settlement, and that certain discrepancies between experiments on the choice of algae are to be explained in this way. Providing that the texture is suitable, the larva will explore to find a groove or depression for permanent attachment.

Age of the frond

Larvae of *A. polyoum* and *Flustrellidra* were offered pieces of *F. serratus* thallus of equal area cut from four different regions. These were frond tips, the region just below the tips ('sub-tips'), and pieces from the centre and the base of the frond, the latter being largely composed of stipe. The pieces of thallus were regularly spaced out in glass finger-bowls, and the dishes arranged in a regular block. Twenty-five larvae were added to each dish and the settlement noted after 24 hr. (Table 9).

Table 9. *The influence of frond age on the settlement of polyzoan larvae on* Fucus serratus

Polyzoan	Region of *F. serratus* frond (pieces of equivalent area)			
	Tip	Sub-tip	Centre	Base
Alcyonidium polyoum	177	133	88	38
Flustrellidra hispida	52	45	64	26

The results clearly show that for *A. polyoum* the tips of the fronds are the most favourable for settlement, and the older the thallus the less favourable it is. The results for *Flustrellidra*, although based on a smaller number of larvae, do not show this trend, and only the base of the frond appears unfavourable. These results appear to reflect a difference in the ecology of these species, and may help to explain how the two co-exist on the same algal substrate. These preferences are clearly important in considering choice experiments, and stress the importance of using pieces of thallus of comparable age. Thus in the choice experiments frond tips were always

used, except with the species of *Laminaria* which have an intercalary growing zone at the base of the thallus; in this case pieces were again cut from the youngest part.

Mucus

Mucus probably plays an important part in determining settlement preferences. Cut surfaces of thick, older *Laminaria* fronds exude large quantities of mucus, but the exudation is much less from young, thin fronds. This was another reason for using the latter in the choice experiments. It seemed important, however, to make sure that settlement, particularly that of *Celleporella* larvae on *L. saccharina*, was not being adversely influenced by the presence of cut edges. Two pieces of thallus, each about 10 cm. long, were cut from young *L. saccharina* fronds. Transverse cuts reaching to the middle were made at intervals of 1 cm. along one side. After thorough washing the two pieces were placed in glass finger-bowls and *Celleporella* larvae were added. In the two experiments the number of larvae that settled was 55 and 145 on the uncut halves and 69 and 49 on the cut halves. Thus on the second piece settlement appeared to be reduced by the cuts, but not on the first. The effect could be due to the interruption of the exploratory behaviour of the larvae by the cuts as well as to the exudation of mucus; the figures indicate that in the choice experiments there may possibly have been a slight adverse effect on settlement on pieces of *Laminaria*.

It has been suggested that in the brackish waters of the Baltic the heavy settlement of epiphytic animals—polyzoans, serpulids and tunicates—on the *Laminaria* fronds is partly due to a reduced mucus content in the thalli (Bock, 1954). The preference of *Alcyonidium hirsutum* larvae for non-fruiting rather than fertile frond tips of *F. serratus* was mentioned earlier. As tufts of paraphyses may be present on non-fruiting fronds, where they do not depress settlement, as well as on fruiting tips, it seems most likely that it is the mucus produced by the conceptacles which accounts for the reduced favourability of fruiting tips.

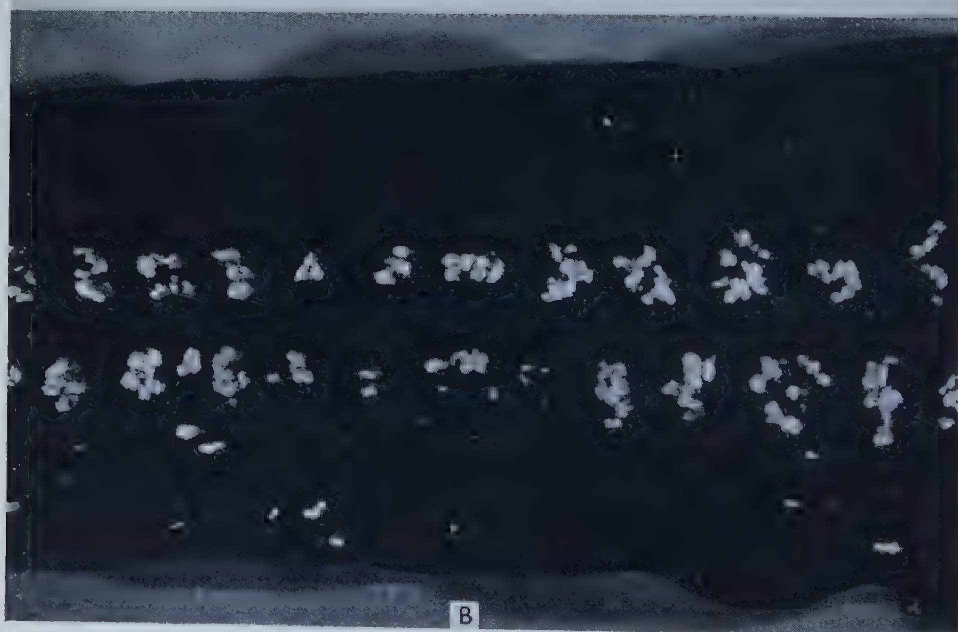
SUMMARY

1. Many species of Polyzoa show marked specificity with regard to the substrate on which they occur. Epiphytic forms are often found mainly on one species of alga.

2. Experiments were performed in which a number of algal species were offered to polyzoan larvae as substrates for settlement. The disposition of algae, and the dishes containing them, was such that the layout conformed to a Youden Square design. This not only achieved economy of materials, but ensured a balanced experiment, made possible a statistical analysis of the results, and eliminated any possible effects of extraneous environmental factors.

3. The larvae showed marked substrate preferences when settling. In the littoral forms *Alcyonidium hirsutum*, *A. polyoum* and *Flustrellidra hispida*, the selection of algae accorded closely with their observed natural distributions: in each case highest settlement took place on *Fucus serratus*. It seems probable that positive selection plays an important role in determining the distribution of these species on the shore. *Celleporella hyalina* larvae were also selective, but the preferences were less clearly related to the ecological distribution of the adult.

A



B

RYLAND—EXPERIMENTS ON THE SELECTION OF ALGAL SUBSTRATES BY POLYZOAN LARVAE

(*Facing p.* 63)

4. Surface texture appears more important than contour as a factor influencing the choice made by larvae between algal substrates, although the physical and/or chemical factors responsible for the observed differences in attractiveness of algae are largely unknown. However, it is evident that the nature of the surface alters with age, and that this influences favourability. The presence of mucus has an adverse effect on settlement. Once the actual substrate has been chosen, the larvae respond to surface contour and, if possible, select a groove or concavity as a site for fixation.

I should like warmly to thank Dr O. L. Davies of Imperial Chemical Industries Ltd for valuable communications relating to the method of analysis of variance employed in this paper; Dr D. J. Crisp for his interest in this work and particularly for advice on statistical procedure and for reading and criticizing the manuscript; and Mr A. P. Austin for information on algal ecology. I am indebted to the Development Commission for a research grant.

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EXPLANATION OF PLATE

The influence of surface contour on the settlement of the polyzoan *Celleporella hyalina* on the frond of *Laminaria saccharina*. One-half natural size. (*A*) Frond of *L. saccharina* showing the mainly flat margins and the strongly crinkled central area—a depression on one side will correspond to a convexity on the other. (*B*) Young colonies of *Celleporella* grouped in the concavities of a frond of *L. saccharina*.

THE ROLE OF THE EPIDERMAL CELLS IN THE 'MIGRATION' OF TRACHEOLES IN *RHODNIUS PROLIXUS* (HEMIPTERA)

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During the moulting process the tracheal system grows by the extension of new tracheae and tracheoles from the existing tubes. Outgrowths are produced by the active movements of the formative cells of the tracheal epithelium; these give out filiform 'amoeboid' processes which move into those regions most deficient in oxygen (Wigglesworth, 1954).

Changes in the distribution of the tracheae can take place also in the absence of any growth. If an extensive area of the epidermis in the non-moulting insect is deprived of its oxygen supply by cutting a main trachea, the tracheoles from adjacent regions 'migrate' in to relieve the deficiency. Many attempts were made to discover the mechanism of this movement, in which considerable forces of traction are evidently involved. But none of the ordinary staining methods employed revealed the strands responsible for the tension on the tracheoles; and it was assumed that amoeboid processes from the cytoplasm of the tracheoles were again concerned—although surprise was expressed at the apparent magnitude of the force they were able to exert.

It was supposed that the tips of the tracheoles are 'attracted' to regions deficient in oxygen. That seems a reasonable assumption when new tracheal cells containing no air-filled tubes are growing outwards during the moulting process. But the tracheoles (in the *Rhodnius* epidermis) contain air right up to their extremities; and they carry this air with them as they 'migrate'.

This thought suggested the possibility that the force of traction might be exerted, not by the cytoplasm of the tracheole, but by the epidermal cells which are in need of oxygen; and that in the non-growing insect the tracheoles might play only a passive role in the movement. The main object of the present work was to test this possibility.

METHODS

Staining of the epidermis

The fourth-stage larva of *Rhodnius* was again used for all the experiments. None of the standard methods of staining that have been tried will reveal cytoplasmic filaments in the epidermis. The modified haematoxylin method that was used for demonstrating the growing axons from the sense cells has been partially successful

(see Wigglesworth, 1953, fig. 15*B*). But methods based on the use of osmium tetroxide and ethyl gallate (Wigglesworth, 1957) have been more satisfactory.

Fixation with 1% buffered osmium tetroxide for 3–4 hr., followed by ethyl gallate (saturated solution in water) for 24 hr., gives excellent staining of mitochondria, but cytoplasmic filaments are generally invisible. On the other hand, if the tissues are first fixed in Bouin's aqueous fixative for half an hour, and then treated with osmium tetroxide and ethyl gallate, although the mitochondrial staining is poor, the cellular filaments are well seen.

Injection of the tracheal system

The use of osmium tetroxide and ethyl gallate has provided the basis for an improved method of injecting the tracheoles. This consists in filling the tracheal system with an unsaturated lipid (using the method of Wigglesworth, 1950) and fixing in osmium tetroxide (4 hr.) followed by ethyl gallate (one day) and mounting in Farrants's gum medium containing ethyl gallate. Olive oil proved too viscous to fill the system completely. Linoleic acid fills the greater part of the system but rapidly penetrates the lining cuticle, forming copious droplets of oil in the cytoplasm beneath (Wigglesworth, 1942). Olive oil mixed with an equal volume of light petroleum oil ('odourless distillate' or kerosene) was wholly successful.

This method gives a completely homogeneous blue-black coloration in the tracheoles, the rounded endings of which are readily seen. The contents have not the finely granular appearance sometimes given by the cobalt sulphide method (Wigglesworth, 1950) and the preparations are completely permanent. Tissues injected and fixed in this way can be cut in sections by the method described elsewhere (Wigglesworth, 1959) and these provide simultaneous demonstration of tracheoles and mitochondria.

It has often been noted that the application of oil to the surface of the cuticle in *Rhodnius* causes this to expand and become corrugated. The same thing happens in the tracheal system. The tubes expand in the long axis and become waved where in the normal state they are straight or smoothly curved. This effect is negligible after fixation directly in osmium tetroxide followed by ethyl gallate. It is very much more evident if the tissues are fixed in Bouin's mixture before treatment with osmium tetroxide. In these preparations the tracheoles, even during 'migration' when they are known to be quite straight and taut, follow a spiral course. So long as this is recognized as an artifact it does not interfere with the observations.

TRACHEAL STRUCTURE

At each moult in *Rhodnius* a new trachea with terminal tracheoles is formed by the outgrowth of tracheal cells. In the final stages the lining of the old trachea is shed, but the linings of the existing tracheoles are not shed—apart from the short segment, only one or two microns in length, which runs between the old trachea and the expanded cuticle of the new trachea (Fig. 1A, B).

Since there is no structural continuity between the new tracheal cuticle and the cuticle of the old tracheole, the question arises as to how these structures are held

together. They are in fact united by a ring of 'glue'. The nature of this adhesive material is not known, but it stains conspicuously with osmium–ethyl gallate and appears as a dense black ring where the old tracheole joins the new tracheal wall (Fig. 1C).

Fig. 1D shows the appearance of these rings as seen in actual specimens. They are present, of course, only at the points where moulting has occurred; there are no such rings at the newly formed extremities of the tracheal system where moulting has not yet taken place.

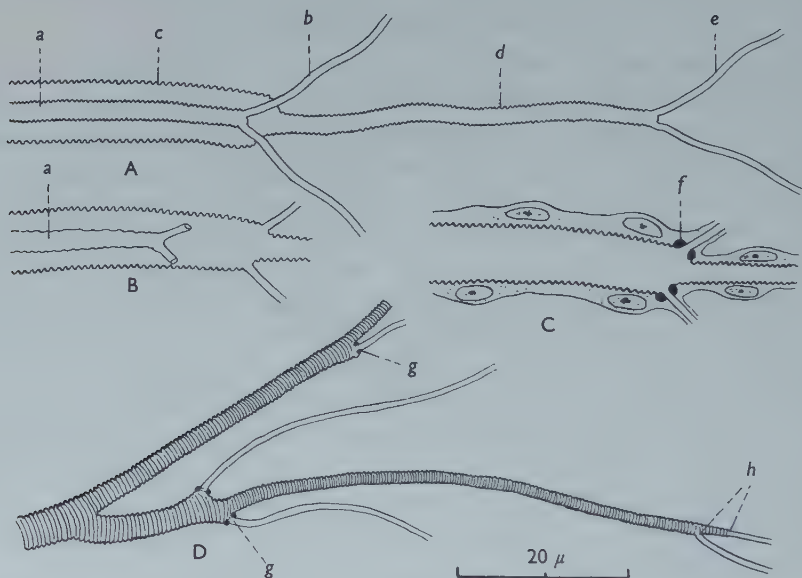

Fig. 1. A, diagram of tracheal ending, shortly before moulting. *a*, old tracheal cuticle; *b*, old terminal tracheoles; *c*, new tracheal cuticle; *d*, newly-formed terminal trachea; *e*, new terminal tracheoles. B, the same at the moment of moulting. The old tracheal cuticle (*a*) with short segments of tracheole attached is being withdrawn. C, the same showing the tracheal epithelium and the rings of cement (*f*) securing the old tracheoles to the new tracheal cuticle. D, actual tracheae and tracheoles in *Rhodnius*. *g*, shows tracheole attachments where moulting has occurred; *h*, shows attachments of recently formed terminal tracheoles.

MECHANISM OF TRACHEAL MIGRATION

Fig. 2 shows the normal distribution of tracheoles below the epidermis in the recently fed 4th-stage larva of *Rhodnius*, along the border between the third and fourth abdominal tergites. The tracheoles follow a convoluted course, but they are rather evenly distributed over the surface (between the basement membrane and the epidermal cells) in such a way that few epidermal cells are separated from a tracheole by more than two or at most three cell widths, that is, a maximum of about 30 μ.

The experiments on tracheal migration have been carried out as described in the earlier paper (Wigglesworth, 1954). The oxygen supply to the right half of the fourth tergite has been eliminated by cutting the main trachea to that segment

close to its origin. The tracheal system has then been injected, and the tergites removed and mounted on successive days after the operation. The tracheoles move in from the third and fifth tergites and from the opposite half of the fourth tergite. Within a week or 10 days they will have moved 700 μ or more from their starting-points.

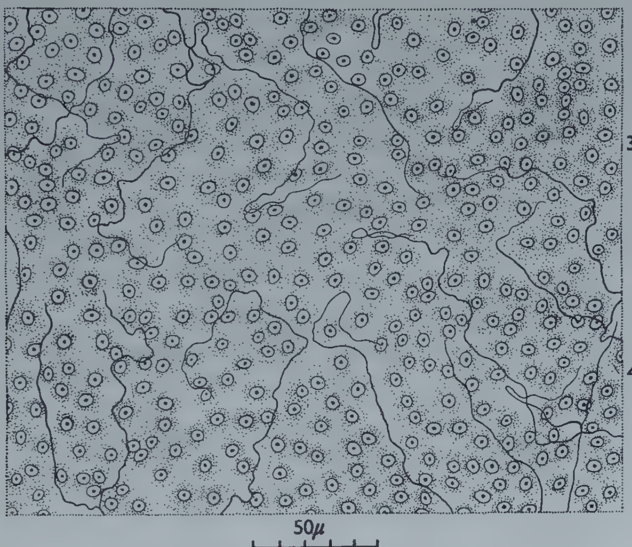

Fig. 2. Distribution of epidermal cells and tracheoles in 4th-stage larva of *Rhodnius* (2 days after feeding) at the junction between the third and fourth tergites.

In the later stages of this process, as already described, the tracheoles run far into the fourth segment, with their tips leading. But in the early stages, at 24 or 48 hr. after tracheal section, the tracheoles are advancing in many different ways. In a few the tip of the tracheole is leading; but in most it is a tracheole loop which is advancing.

The speed of movement of the tracheoles depends upon the stage of growth of the epidermal cells. If the trachea is cut at 24 hr. after feeding, particularly if the insect is decapitated so that growth fails to begin, the epidermal cells are greatly attenuated and the movement is relatively slow. But if the trachea is not cut until 5 days after feeding, when the epidermal cells have enlarged to form a thick cubical epithelium, the movement is more rapid, and within 2 or 3 days many of the tracheoles will have extended half-way to the middle of the fourth tergite.

The first indication that the epidermal cells might be responsible for the tracheole movement was obtained in preparations fixed and stained with osmium tetroxide and ethyl gallate. Fig. 3A shows the cells in the neighbourhood of an advancing tracheole loop. In this insect the trachea was cut at 5 days after feeding and the preparation made 1 day later. No strands or filaments from the epidermal cells were visible; but it was obvious that almost every cell in the neighbourhood of the air-filled tracheoles had most of the mitochondria on the side of the nucleus that was directed towards the tracheole, and many of the mitochondria were oriented

in that direction. This appearance suggested that invisible strands were being extended to the tracheole.

Clear proof of this was obtained when the epidermis was fixed in Bouin's mixture before treatment with osmium tetroxide. Fig. 3 B shows the tracheoles advancing into the fourth tergite of an insect in which the trachea to that segment had been cut at 1 day after feeding and the preparation made 4 days later. Each of the advancing tracheoles has a group of exceedingly fine straight filaments converging upon it. Sometimes these filaments are attached to loops of tracheoles, sometimes to the tracheole tip. These are clearly the filaments which are drawing the tracheoles along.

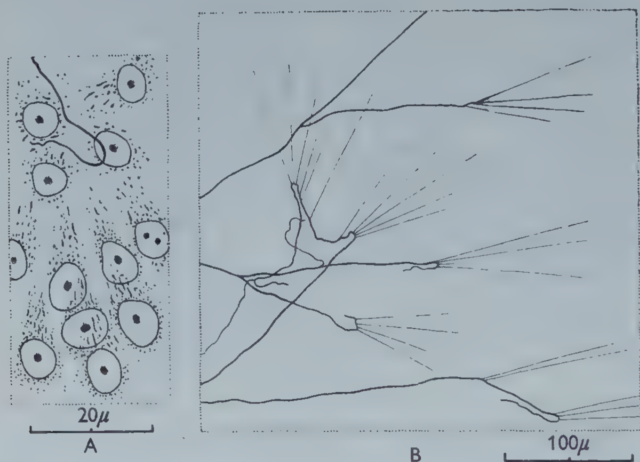

Fig. 3. A, epidermal cells 1 day after tracheal section showing orientation of mitochondria towards air-filled tracheole. Osmium, ethyl gallate. B, tracheoles of adjacent segment advancing into fourth tergite, 4 days after tracheal section, showing the filaments responsible for the traction. Bouin, osmium, ethyl gallate.

Fig. 4 shows in detail one of the advancing loops represented in Fig. 3 B. It can now be seen that all the filaments arise from conical processes given out by the epidermal cells. Indeed, when the specimen is closely examined scarcely a cell is to be found, within a radius of 100–125 μ from the tracheole, which is not connected to it by a strand. Many of these strands are exceedingly fine; they are necessarily shown too thick in the figure.

Fig. 5 illustrates further details of the epidermal cells and filaments. Fig. 5 A is from a 4th-stage larva in which the trachea was cut at 1 day after feeding and the specimen injected and mounted 3 days later. A single distinct strand from the tip of an advancing tracheole has been followed to its origin in darkly staining cones arising from two epidermal cells about 100 μ away; but it is probable that other cells also contribute to this strand.

Fig. 5 B is from a larva with the trachea cut at 1 day after feeding, injected and mounted 4 days later. A group of epidermal cells 60–80 μ from the tracheole loop are sending out conical tapering processes. Two of these cells are drawn in detail; their filaments extend to the tracheole without fusing.

Fig. 5C is from a larva with the trachea cut at 5 days after feeding, injected and mounted 2 days later. By that time (48 hr. after tracheal section) the tracheoles had already moved about 200μ from their starting-point. The drawing shows two epidermal cells sending out darkly staining processes in the direction of an advancing tracheole loop about 90μ distant. These outgrowths have not yet made contact

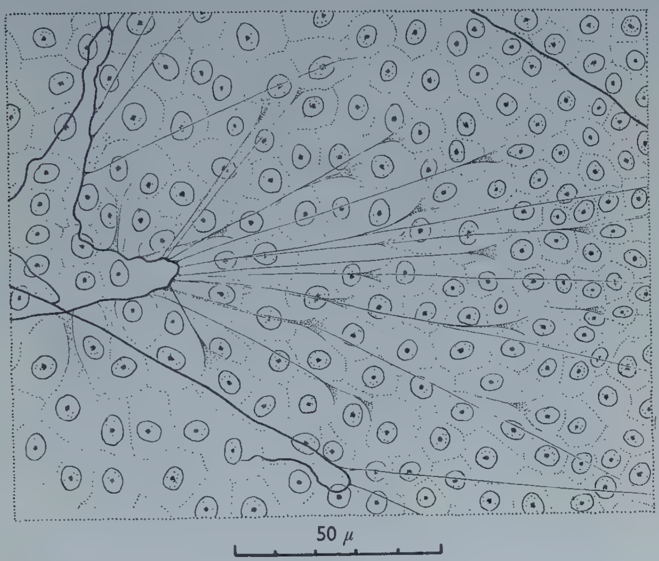

Fig. 4. Detail of two of the tracheole loops seen in Fig. 3B, showing filaments connected with the epidermal cells.

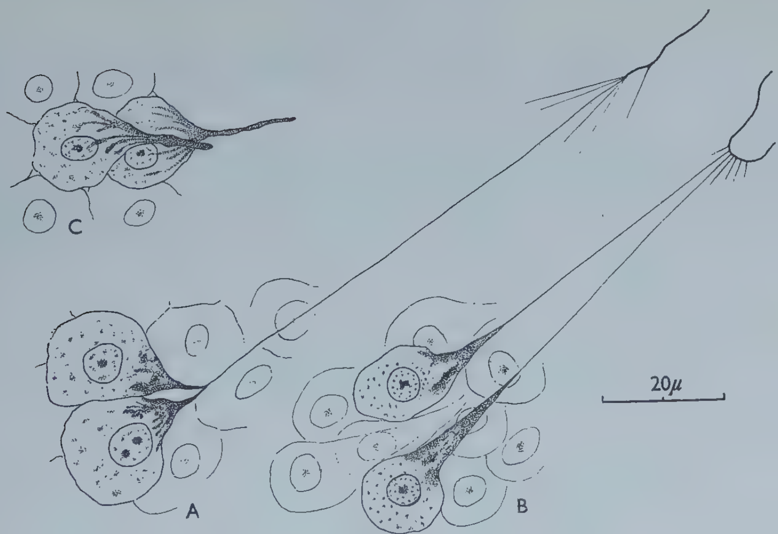

Fig. 5. A, two epidermal cells forming a filament attached to a tracheole; 3 days after tracheal section. B, similar preparation; 4 days after tracheal section. C, two epidermal cells sending out darkly staining processes in the direction of a tracheole loop about 90μ distant. Bouin, osmium, ethyl gallate.

with the tracheole to form filaments. They presumably represent an early stage in the formation of such filaments.

As we have already seen, the filaments from a number of epidermal cells may fuse to form composite strands, which may sometimes be as much as 1μ in thickness. Fig. 6 is from a specimen in which the trachea was cut at 1 day after feeding, injected and mounted 6 days later. In this figure a group of advancing tracheoles, with their tips leading, lies about 40μ beyond the right-hand margin of the drawing,

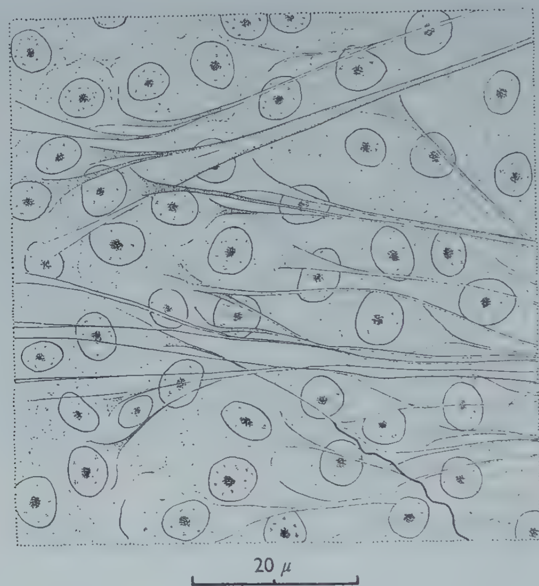

Fig. 6. Epidermal cells with filaments fusing to form relatively thick strands which are connected to tracheoles about 40μ to the right of the figure.

and the limit of the epidermal cells that are reacting lies about 50μ beyond the left-hand margin. The total distance from the tips of the tracheoles to the limit of reacting cells is thus about 150μ. In the area shown cytoplasmic extensions are visible from practically every epidermal cell; many of these processes are fusing to form composite strands.

REACTION OF THE EPIDERMAL CELLS TO REDUCED CONCENTRATIONS OF OXYGEN

In a further series of experiments 4th-stage larvae at different times after feeding were transferred to nitrogen containing 1% or 4% of oxygen. For the most part the epidermal cells lie so close to the tracheoles that they cannot form the very long filaments described above. But they do show an undoubted response to the tracheoles. Whereas in the control insects kept in air, only an occasional strand of cytoplasm from an epidermal cell is attached to a tracheole, in the insects exposed to reduced oxygen, cytoplasmic strands converge upon the tracheoles from all sides.

Fig. 7 is from a larva which was transferred to 4% oxygen in nitrogen at 5 days after feeding, injected and mounted 1 day later. It shows an area where there happen to be three tracheole loops rather widely separated. Most of the epidermal cells are sending out cytoplasmic strands which are attached to the tracheoles.

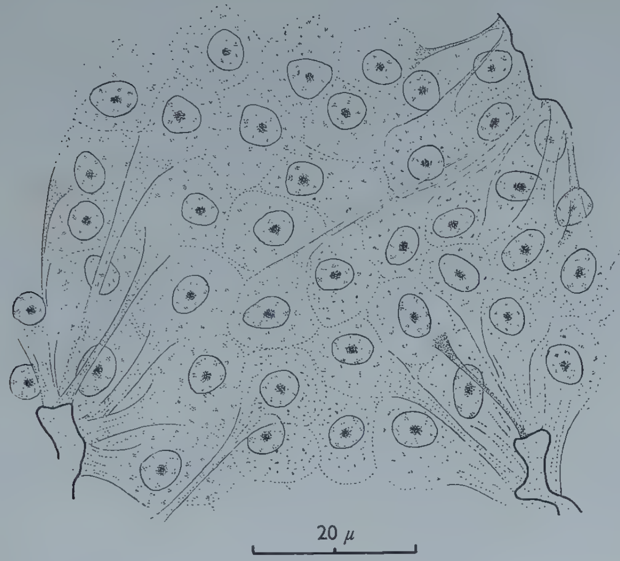

Fig. 7. Epidermal cells of a larva transferred to 4% oxygen at 5 days after feeding and mounted 1 day later, showing cytoplasmic strands attached to three tracheoles.

DISCUSSION

The observations described clearly prove that in the non-moulting insect it is the epidermal cells which provide the physiological response and the mechanical force which pulls the air-filled tracheoles into the oxygen-deficient regions. Whether the same response is given by other tissues has not been proved. The distribution of tracheoles in the fat body is so variable that it has not been possible to see whether any migration takes place.

If a wound is made in the integument of *Rhodnius* the epidermal cells in the surrounding zone leave their attachments to the cuticle and migrate to the margin of the injury, creating a peripheral zone of sparse cells (Wigglesworth, 1937). The reaction of the oxygen-deficient epidermal cells is strikingly different. There is no migration of cells and no aggregation around the tracheoles. The cells merely send out processes which draw the tracheoles towards them.

While these processes are making their way towards an air-filled tracheole, they may encounter one of the tracheoles from the trachea that has been cut. It is not uncommon for them to attach themselves to this tracheole and to pull it out into a loop towards them. Whether this is a purely mechanical reaction, or whether the cut tracheole does in fact contain sufficient oxygen to provoke the response is uncertain.

In any case it is clear that the traction of the epidermal cells upon the tracheoles is a normal phenomenon in the physiology of the epidermis. Cytoplasmic strands from the cells to the tracheoles can be seen from time to time in the normal insect. It is presumably this traction which is responsible for the even distribution of the tracheoles (Fig. 2).

Of particular interest is the great distance (100–150μ) over which the epidermal cell will respond to the presence of an air-filled tracheole and send out a filament towards it. These filaments appear to be formed from a material which takes up osmium only after fixation with picric acid. This material is concentrated in the cone which gives rise to the filament. Its nature is unknown; but it is worth noting that the mitotic apparatus in the dividing cells of the epidermis shows a similar staining reaction.

These long slender fibrils, which sometimes appear to fuse with the fibrils of adjacent epidermal cells, are evidently contractile structures under the control of the cell from which they arise. When their task is accomplished they are absorbed again into the cytoplasm.

SUMMARY

1. The tracheal system can be injected with a mixture of olive oil and light petroleum, and fixed with osmium tetroxide followed by ethyl gallate. This provides simultaneous demonstration of tracheoles and mitochondria.

2. During moulting in *Rhodnius* the lining of the tracheoles (which is not shed) is joined to the new cuticle of the trachea by a ring of cement substance which stains deeply with osmium and ethyl gallate.

3. The epidermal cells are responsible for the 'migration' of air-filled tracheoles into regions deprived of their normal tracheal supply. The cells give rise to contractile filaments, sometimes 100μ in length, which become attached to the tracheoles and draw them inwards.

4. Contractile cytoplasmic strands from the epidermal cells probably ensure the even distribution of the tracheoles. They become much more numerous if the insect is exposed to a low concentration of oxygen.

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STUDIES ON THE EXCHANGE AND REGULATION OF SODIUM IN THE LARVA OF *AËDES AEGYPTI* (L.)

I. THE STEADY-STATE EXCHANGE

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I. INTRODUCTION

The larva of *Aëdes aegypti* (L.) lives naturally in fresh waters in which it keeps the concentration of salts in its haemolymph far above that in the medium. It does this by secreting salts from the medium into the haemolymph against a steep concentration gradient by means of the anal papillae, and by resorbing salts from the urine through the walls of the rectum; the Malpighian tubules also contribute to the work of salt retention by secreting a urine which is slightly hypotonic to the haemolymph (Ramsay, 1950, 1951, 1953). The larvae can keep the levels of sodium, potassium and chloride in the haemolymph constant despite wide variations in their concentrations in the medium (Wigglesworth, 1938; Ramsay, 1953). The anal papillae are responsible for most of the permeability of the larva to salts and water (Wigglesworth, 1933*a*, *b*; Treherne, 1954).

The steady-state exchange of sodium between the starved larva and the medium has been studied by Treherne (1954). The exchange appears to be a first-order process and it occurs mainly through the anal papillae. It is independent of the potassium concentration in the medium over the range 0·159–4·0 mM/l., and of the sodium concentration over the range 4–8 mM/l.

In this paper I shall describe experiments on the steady-state exchange of sodium between the haemolymph (or whole larva) and the medium. ^{22}Na has been used to study the exchange. The sodium in the haemolymph has been measured by equilibrating or rearing larvae in labelled media, and by flame photometry using an 'Eel' flame photometer. Experiments on the regulation of the sodium level in the haemolymph will be described in a further paper. The aim throughout the work was to try to throw more light on the processes of sodium exchange and regulation by altering suitably the conditions to which the larvae were exposed.

II. MATERIALS AND METHODS

Larvae were reared from the eggs in lots of 150–300 in 500 ml. of the following medium:

	mM/l.		mM/l.
NaCl	2·000	$MgCl_2$	0·200
KCl	0·500	KOH	0·059 approx.
$CaCl_2$	0·500	KH_2PO_4	0·100 approx.

The pH was adjusted to about 6·3; 0·3 g. of a mixture of dog biscuit and 'Bemax' was added and the resulting infusion provided a comfortable excess of food up to the 4th instar and beyond. Larvae reared in this way moulted into the 4th instar within a few hours of one another. The addition of the foodstuff to the medium caused an increase in the sodium, potassium and calcium contents of about 1, 13 and 5 % respectively.

Both fed and starved 4th instar larvae were used in the experiments. The fed larvae were always given an excess of food, and the starved larvae were kept in a medium similar to the one they were reared in but without food for at least 60 hr. before the start of an experiment. I have found that larvae which have recently moulted into the 4th instar can withstand 9–12 days of rigorous starvation before any die. All the experiments were done at 28° C. and the media were aerated with compressed air.

To study the exchange of sodium between the larvae and the medium the larvae were reared in unlabelled or labelled media, starved if necessary, and put (after appropriate washing) into 500 ml. of labelled or unlabelled media. The alteration in radioactivity in the haemolymph was noted at appropriate intervals. In the case of fed larvae the experimental media contained 0·1 g. of foodstuff. When the uptake of radioactivity was followed the total sodium content was found by flame photometry. In some cases the exchange of sodium in single larvae was studied. For this the larvae were reared in labelled media and put into unlabelled media. They were removed from these at appropriate intervals, dried with filter paper, and put on a marked area on a special counting disk. The radioactivity was measured for 2–4 min. and the larvae were returned to the media. It was assumed that self-absorption of radioactivity stayed constant. For measurements of radioactivity haemolymph was collected (under a binocular microscope) from each of a group of five larvae into silicone-lined glass braking-pipettes 0·1–0·2 μl. in volume. The larvae had previously been dried with filter paper. The haemolymph was then placed, with the washings, on separate counting disks and after it had dried it was assayed for radioactivity. For each group the mean radioactivity per μl. and its standard deviation were calculated. Radioactivity adhering to the surface of the larvae was, if necessary, removed before collecting the haemolymph by placing them in a slow stream of tap water for 1 min. The radioactivity was measured with G.E.C. GM4 end-window counters and appropriate scaling equipment. Because of the long half-life of ^{22}Na (2·6 years) it was not necessary to correct the measurements for radioactive decay. The accuracy of the measurements was as follows:

(*a*) Radioactivity of the whole larvae; the standard deviation varied from 2·6 % at the start of an experiment to 10 % at the end when the radioactivity was very low.

(*b*) Radioactivity of haemolymph samples; for weak samples the standard deviation was between 4·8 and 7·1 %, for active samples it was between 1·6 and 3·2 %. The pipettes were calibrated with a solution of ^{22}Na of known radioactivity. The standard deviation of their volumes was 1·4 %. Several pipettes were used during each experiment as a deposit from the haemolymph slowly forms on the silicone lining. ^{22}Na was obtained as a practically weightless solution of the

chloride from the Radiochemical Centre, Amersham. The specific activities of the labelled media were between 1·5 and 5·4 mc. ^{22}Na/g. Na.

For measurement of the sodium level in the haemolymph by flame photometry 0·1–0·2 μl. of haemolymph was collected from each of a group of five larvae and placed, with the washings, in a small polythene tube containing 5 ml. of distilled water. The larvae had previously been washed in a large volume of distilled water and dried on filter paper. The tube was then stoppered and shaken and the sample analysed on the 'Eel' flame photometer using standard solutions containing up to 1·7 parts per million of sodium (as chloride). At least three estimations were made on each sample and the mean and its standard deviation were calculated. In this way the sodium in solutions containing roughly 0·1–0·5 μg. Na/ml. could be analysed with an accuracy of $\pm$1–4% (standard deviation).

Equations describing the steady-state exchange of an ion between a single compartment system and an infinite amount of external medium are given by Harris & Burn (1949). For a system equilibrated in an unlabelled medium and then put into a labelled one the equation is

$$-\mathrm{K}_{\mathrm{out}}t = \ln\left(1 - \frac{[\mathrm{Na}^*_{\mathrm{in}}]_t}{[\mathrm{Na}^*_{\mathrm{in}}]_\infty}\right), \tag{1}$$

for a system equilibrated in a labelled medium and then put in an unlabelled one the equation is

$$-\mathrm{K}_{\mathrm{out}}t = \ln \frac{[\mathrm{Na}^*_{\mathrm{in}}]_t}{[\mathrm{Na}^*_{\mathrm{in}}]_{t=0}}, \tag{2}$$

where

$\mathrm{K}_{\mathrm{out}}$ = permeability constant direction in $\rightarrow$ out,
$[\mathrm{Na}^*_{\mathrm{in}}]_t$ = labelled sodium in the system at a given time,
$[\mathrm{Na}^*_{\mathrm{in}}]_\infty$ = labelled sodium in the system at infinite time,
$[\mathrm{Na}^*_{\mathrm{in}}]_{t=0}$ = labelled sodium initially present in the system.

These equations may be formulated in terms of the flux of sodium instead of the permeability constants (Croghan, 1958*b*). The flux may be found from the relationship

$$m = \mathrm{K}_{\mathrm{out}}\,[\mathrm{Na}_{\mathrm{in}}], \tag{3}$$

where m = the flux of sodium in either direction,
$[\mathrm{Na}_{\mathrm{in}}]$ = the concentration of sodium in the system.

The time for half exchange ($T_{\frac{1}{2}}$) of the sodium in the system may be found from the relationship

$$\mathrm{K}_{\mathrm{out}} = 0{\cdot}693/T_{\frac{1}{2}}. \tag{4}$$

Where the area and volume of the system are not known but are assumed to be constant (as in this work) the permeability constant $\mathrm{K}_{\mathrm{out}}$ has the dimension of 1/time. It is related to the true permeability constant which has the dimension of distance/time in the following way:

$$\mathrm{K}_{\mathrm{out}} = \mathrm{K}'_{\mathrm{out}}\,A/V, \tag{5}$$

A and V being the area and volume of the system and $\mathrm{K}'_{\mathrm{out}}$ the true permeability constant.

K_{in} (the permeability constant out $\rightarrow$ in) is obtained by substitution in the equation

$$\frac{K_{in}}{K_{out}} = \frac{[Na_{in}]}{[Na_{out}]}, \tag{6}$$

where $[Na_{out}]$ is the concentration of sodium in the medium.

For a many-celled animal like a mosquito larva the permeability constants describe only the overall process of permeation. The curves described by equations (1) and (2) will from now on be called 'exchange uptake' and 'exchange washout' curves. Plots of the logarithmic expressions of these equations against time give straight lines the slopes of which are equal to K_{out}. The error in determining the points increases as the exchange nears completion (Solomon, 1952) so it is not legitimate to calculate regression lines in order to find K_{out}. Following Treherne (1954) K_{out} is here found as the mean of the slopes of the lines joining the individual points to the origin.

III. RESULTS

In what follows the term 'normal larvae' refers to larvae either fed or starved which have not been operated on.

It has been found that the sodium level in the haemolymph of normal larvae is not reduced by starvation for periods greater than 200 hr., but remains steady at about 100 mM/l. The sodium level in fed larvae also remains steady at about this level up to the time of pupation.

Results for the exchange of sodium in fed and starved normal larvae are shown in Figs. 1 and 2. It is clear that the exchange proceeds much more rapidly in fed than in starved larvae, and that all the sodium in the haemolymph (and whole larva) is exchangeable with that in the medium. When the values for

$$\log_{10}\left(1 - \frac{[Na^*_{in}]_t}{[Na^*_{in}]_\infty}\right) \quad \text{and} \quad \log_{10}\left(\frac{[Na^*_{in}]_t}{[Na^*_{in}]_{t=0}}\right)$$

are plotted against time satisfactory straight lines are obtained (Fig. 3) showing that the exchange follows equations (1) and (2).

During the course of an experiment with starved larvae the pH of the medium rises slowly from 6·3 to about 7·2, but this does not appear to affect the exchange of sodium which proceeds exponentially throughout the experiment. In the case of fed larvae the pH of the medium is made to change in a complex manner by the addition of the foodstuff and the subsequent growth of microbes. However this change may be ignored as a change of pH from 5·2 to 7·2 (a range greater than that encountered) was found not to affect the exchange. The small amounts of potassium and calcium which were added to the medium with the foodstuff were shown not to alter the rate of exchange in starved larvae. This is to be expected from Treherne's observations.

The information provided by the experiments on normal larvae is given in Table 1. Data obtained from single larvae are not included as these do not

adequately represent the exchange in a population of larvae. The figures marked with an asterisk will not be considered further as they were obtained from larvae put by accident into a medium containing a slight growth of mould.

Table 1 shows that feeding reduces $T_{\frac{1}{2}}$ by a factor of 6·33–7·0.

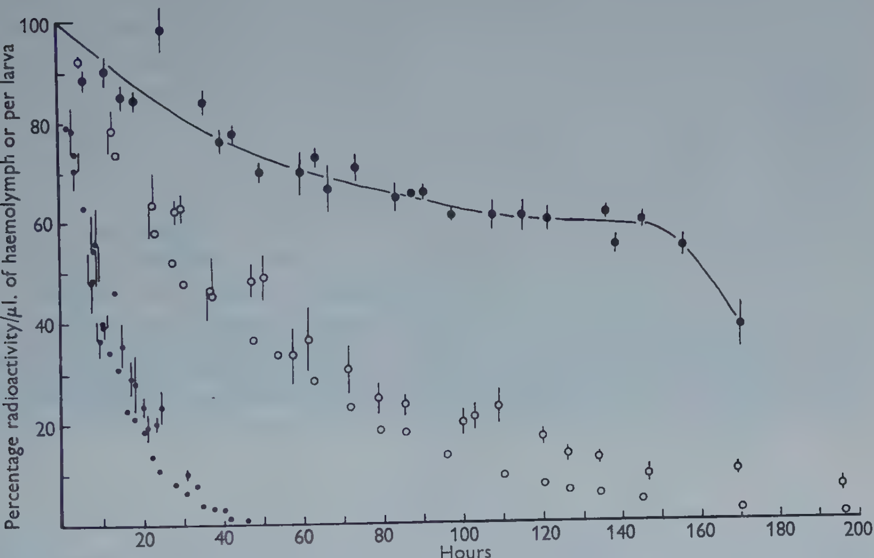

Fig. 1. ● = outflux of labelled sodium from the haemolymph of starved larvae put into flowing distilled water. ○ = exchange washout of labelled sodium from the haemolymph of starved larvae and from a single starved larva. • = as above but for fed larvae. The vertical lines show the extent of the standard deviations. The standard deviations for the results for single larvae are not shown in the figure.

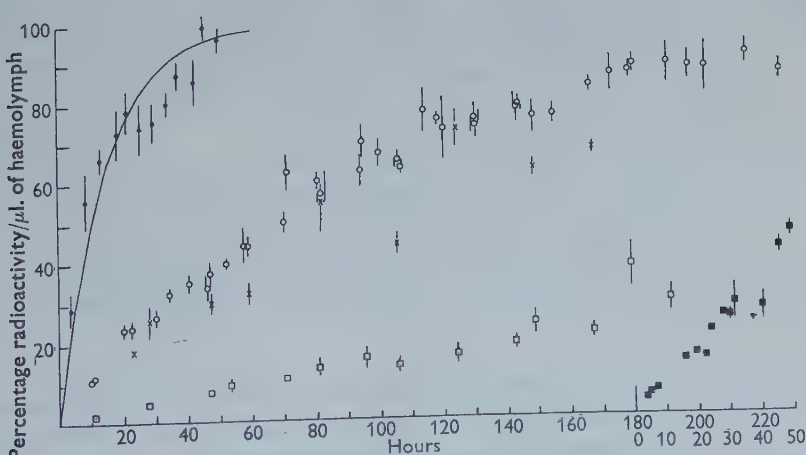

Fig. 2. • = exchange uptake of labelled sodium into the haemolymph of fed larvae. ○ = as above but for starved larvae. × = as above but for starved larvae with the gut blocked with Whitehead's varnish. ■ = exchange uptake of labelled sodium into the haemolymph of fed papilla-less larvae. □ = as above but for starved papilla-less larvae.

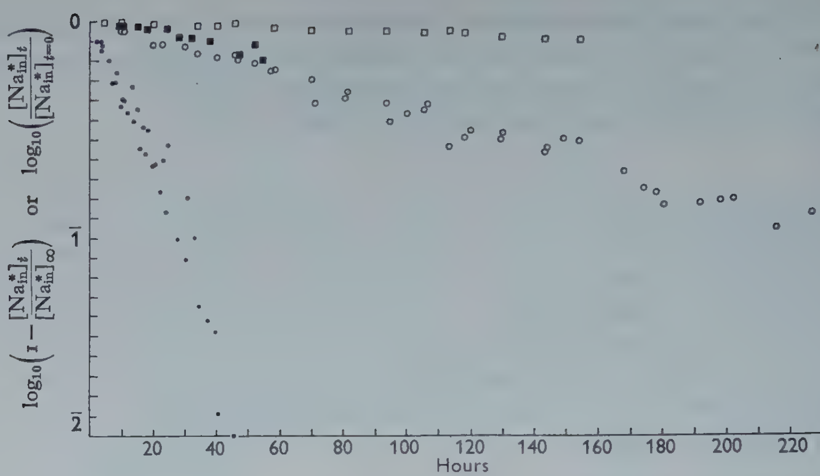

Fig. 3. ● = semi-logarithmic plot for the exchange washout of labelled sodium from the haemolymph of fed larvae and from a single fed larva. ○ = semi-logarithmic plot for the exchange uptake of labelled sodium into the haemolymph of starved larvae. ■ = semi-logarithmic plot for the exchange uptake of labelled sodium into the haemolymph of fed papilla-less larvae. □ = as above but for starved papilla-less larvae.

Table 1

State of larvae	Type of exchange	$[Na_{in}]$ (mM/l. of haemolymph)	K_{in} (hr.$^{-1}$)	K_{out} (hr.$^{-1}$)	$T_{\frac{1}{2}}$ (hr.)	Flux of sodium mM/l. of haemolymph/hr.
Starved	Washout	99·8 (R)	0·809*	0·0162*	42·7*	1·62*
Starved	Uptake	103·6 (FP)	0·562	0·0109	63·5	1·13
Starved	Uptake	96·4 (FP)	0·527	0·0109	63·5	1·05
Fed	Washout	116 (R)	4·42	0·0761	9·1	8·84
Fed	Uptake	103·6 (FP)	3·57	0·0690	10·03	7·15

R = determined by radioactive equilibration at the start of the experiment.
FP = determined by flame photometry during the experiment.
* See text.

For starved larvae the following figures are available:

Medium	K_{out}	K_{in}	
4 mM/l. NaCl	0·012 hr.$^{-1}$	0·288 hr.$^{-1}$	Treherne (1954)
8	0·012	0·150	Treherne (1954)
2	0·011	0·54	Present results, Table 1

It is clear that while K_{out} and $[Na_{in}]$ remain constant, $K_{in} \propto (1/[Na_{out}])$. This means that identical amounts of sodium exchange in the three different media.

The sodium in the haemolymph may exchange with that in the medium through the anal papillae, the gut and the general body surface. As far as the permeation of sodium is concerned the larva may be regarded, as a first approximation, as three resistances in parallel, in which case the sum of the permeabilities of the three tissues

should equal the permeability of the intact larva. An estimate of the exchange occurring through the papillae can be obtained by using larvae which have had the papillae destroyed by 5% NaCl (Wigglesworth, 1933*b*). This treatment makes the sodium concentration in the haemolymph of both fed and starved larvae drop to about 80 mM/l. (see Koch, 1938) but it then remains steady at this level. The exchange uptake of sodium in fed and starved papilla-less larvae is shown in Figs. 2 and 3 and the results are summarized in Table 2. Feeding reduces $T_{\frac{1}{2}}$ by a factor of 3·14–6·6. A comparison of Tables 1 and 2 shows that in the case of fed larvae destruction of the papillae reduces K_{out} to 7·29–14·6% and K_{in} to 4·15–11·7% of the normal value. In the case of starved larvae K_{out} is reduced to 14–16·2% and K_{in} to 11–13·7% of the normal value. The discrepancy between the alteration in K_{out} and K_{in} is due to the drop in haemolymph sodium caused by the destruction of the anal papillae. These results show that roughly 90% of the exchange of sodium occurs through the papillae. This estimate is supported (in the case of starved larvae) by data for the exchange in larvae with the gut blocked by means of a drop of Whitehead's varnish put on the mouthparts. Although these larvae were adversely affected by the varnish the rate of exchange was reduced only by a relatively small amount (Fig. 2). Treherne (1954) showed that the exchange through the general body surface was very small.

Table 2

State of larvae	Type of exchange	$[Na_{in}]$ (mM/l. of haemolymph)	K_{in} (hr.$^{-1}$)	K_{out} (hr.$^{-1}$)	$T_{\frac{1}{2}}$ (hr.)	Flux of sodium mM/l. of haemolymph/hr.
Starved papilla-less	Uptake	81 (FP)	0·0619	0·00153	453	0·124
Starved papilla-less	Uptake	81·4 (FP)	0·072	0·00177	392	0·144
Fed and papilla-less	Uptake	66·0 (FP)	0·183	0·00555	125	0·366
Fed and papilla-less	Uptake	82·6 (FP)	0·416	0·01008	68·8	0·833

To summarize:

(1) Although feeding to satiety does not cause an appreciable alteration in the sodium level in the haemolymph it increases the rate of exchange by a factor of 6·33–7.

(2) Roughly 90% of the exchange in both fed and starved larvae occurs through the anal papillae and the rest occurs through the gut and general body surface. Therefore roughly 90% of the increase in exchange rate due to feeding occurs through the anal papillae.

The outflux of labelled sodium from the haemolymph of starved larvae placed in flowing distilled water is shown in Fig. 1. The larvae are able to retain the sodium to a very considerable extent. At about 140 hr. a rapid drop in the sodium level occurs and the larvae start to die. This experiment confirms and extends information given by Treherne (1954).

Wigglesworth (1933*a*) gives a detailed description of the anal papillae of larvae reared and presumably starved in fresh water. I have found that considerable cytological changes follow upon feeding and starvation. The papillae studied were fixed in Carnoy's fluid and stained with iron haematoxylin and eosin. Papillae of

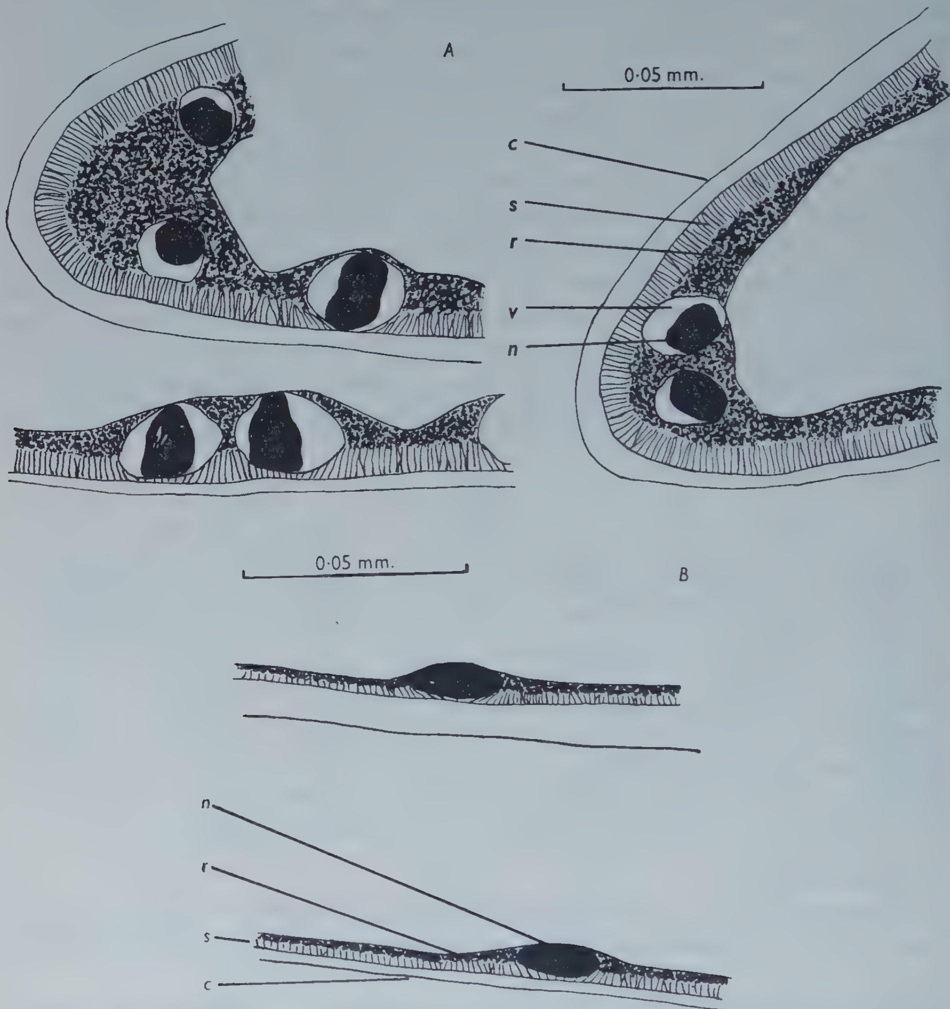

Fig. 4. Semi-diagrammatic drawings made from photomicrographs of fixed and stained anal papillae. The drawings represent longitudinal sections through the papillae. *A* = papillae of fed larvae. *B* = papillae of starved larvae. *c* = cuticle; *s* = striated region of cytoplasm; *r* = reticular region of cytoplasm; *v* = vesicle; *n* = nucleus.

fed larvae are illustrated in Fig. 4*A* and those of starved larvae in Fig. 4*B*. The cytoplasm of fed papillae is much thicker than that of starved ones. The nuclei of fed papillae are usually rather irregular in shape and are found inside large vesicles. This suggests that they have shrunk considerably during preparation and that when

living they are much larger than those of starved papillae. As far as can be judged by studying the surface of the papillae the density per unit area of the striations is about the same in the fed and starved papillae.

IV. DISCUSSION

In larvae reared and kept in the medium the distribution of Na, K and Cl between the haemolymph and the medium is as follows:

	Haemolymph (mM/l.)	Medium (mM/l.)
Na	100	2
K	4·2 (Ramsay, 1953)	0·659
Cl	51·3 (Wigglesworth, 1938)	3·9

Clearly the larva can bring about a very considerable accumulation of these elements. It can also keep the levels of these elements in the haemolymph remarkably constant when their levels in the medium are varied between < 1 and 80–100 mM/l. (Wigglesworth, 1938; Ramsay, 1953). Furthermore larvae made deficient in these elements can restore them to their normal levels in the face of a steep concentration gradient (Wigglesworth, 1938; Ramsay, 1953; Stobbart, unpublished). Because of these facts, and by comparison with the situation in other tissues, it seems likely that Na is actively pumped into the haemolymph. In what follows I shall assume that this is so. I shall also assume, as a first approximation, that the larva consists of only the haemolymph compartment (see Treherne, 1954).

About 90% of the steady-state exchange occurs through the anal papillae and the rest occurs mainly through the gut. It will be shown in a further paper that almost all the net transport by sodium-deficient larvae occurs through the papillae, so it would be simplest to suppose that the steady-state exchange results from an active influx through the papillae which balances a passive outflux through these and other tissues. But by feeding the larvae the fluxes and the exchange rate may be increased by a factor of about 6·5. Consider first the fluxes through the papillae. The relative sizes of the fluxes in fed and starved larvae are shown in Fig. 5*A*. If the outfluxes are due to passive diffusion through leaks in some semi-permeable membrane in the papillae, the fed larvae would have to move 6·5 times more sodium inwards to keep the sodium in the haemolymph at the same level. This seems most improbable. It is unlikely that feeding has a bad effect on the semi-permeable membrane increasing its in → out permeability by a factor of 6·5; it is also unlikely that the electrochemical activity of the sodium inside fed larvae is 6·5 times greater than in starved larvae when its concentration in both is the same. It is more likely that both influx and outflux are mediated by a carrier mechanism which is linked to the metabolism of the larva, and that when the larvae are fed the carrier is altered so that the fluxes are increased. The carrier is assumed to be confined to some osmotic barrier in the papillae between the haemolymph and the medium. The barrier itself is assumed to be largely impermeable to sodium. According to this view, in fed larvae almost all the influx and at least 85% (= 5·5/6·5) of the

outflux are carrier-mediated. In starved larvae almost all the influx is presumably carrier-mediated but there is no evidence as to whether the outflux is carrier-mediated or whether it results from passive diffusion, but presumably some of it results from passive diffusion. This interpretation is shown diagrammatically in Fig. 5*B*; it has been assumed arbitrarily that in starved larvae half the outflux is due to passive diffusion. Treherne (1954) showed that potassium did not compete with sodium for the exchange process, and it has been shown that the exchange is independent of $[Na_{out}]$ over the range 2–8 mM/l. These observations were only made on starved larvae, but it seems probable they would apply to fed larvae as well. The carrier mechanism therefore must be specific to sodium and saturated with it at the external concentration.

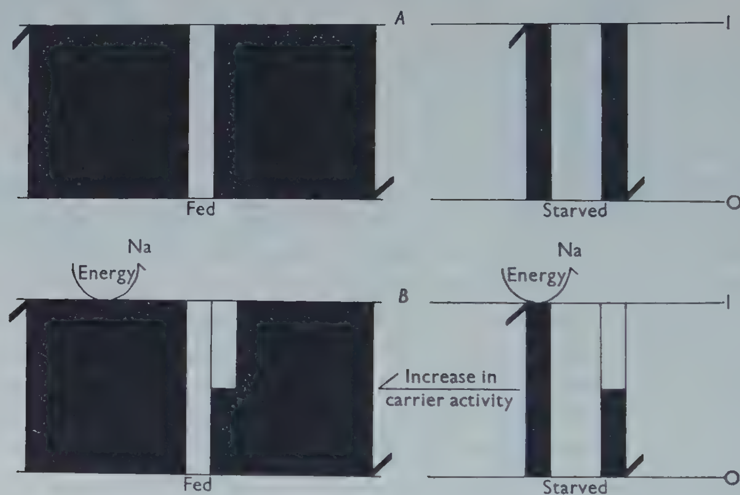

Fig. 5. A = diagram showing the relative sizes of the fluxes of sodium through the anal papillae of fed and starved larvae. In both A and B, I and O represent the inner and outer surfaces of the osmotic barrier, and the thick arrows represent the fluxes of sodium. The relative sizes of the fluxes are represented by the areas of these arrows. B = diagram of the proposed sodium carrier and pump. The black areas of the thick arrows represent the carrier-mediated fluxes of sodium and the white areas represent the passive outflux. The same amount of net carrier-mediated influx is supposed to occur in both fed and starved larvae in order to balance the same amount of passive outflux. Both fed and starved larvae must therefore do the same amount of osmotic work. This is represented by thin arrows of the same size.

The results are most satisfactorily explained in terms of a sodium pump (that is, a source of free energy) working in conjunction with something similar to an exchange diffusion mechanism (Ussing, 1948) which is confined to the osmotic barrier. The pump is supposed to split off at the inner surface of the barrier sufficient sodium from the 'exchange diffusion mechanism' (EDM) to balance any passive loss. The EDM then takes up some sodium from the medium. When the larvae are fed the exchange, but not the pumping of sodium, is increased (Fig. 5*B*). The exchange component of the fluxes may be regarded as an exchange diffusion in that no osmotic work is associated with it. The increase in exchange could be brought

about by (i) an increased rate of movement of the EDMs, (ii) an increase in the amount of sodium carried by each EDM, (iii) a synthesis of more EDMs, (iv) an increase in any possible enzymic catalysis of exchange of sodium between EDM and medium or haemolymph (see Mitchell, 1954*a*, *b*). A similar explanation was offered by Mitchell & Moyle (1953) for the exchange and net transport of phosphate in *Micrococcus pyogenes*. On the other hand Croghan (1958*a*, *b*) found that in *Artemia salina* an ionic pump occurs in the gut and an exchange diffusion mechanism in the general body surface.

It has so far been assumed that influx and outflux through the anal papillae are equal. However when the papillae are removed the sodium level in the haemolymph drops from about 100 to 80 mM/l., and larvae which were reared in the medium are unable to bring back the sodium to 100 mM/l. This suggests that the influx through the papillae is normally somewhat greater than the outflux. This is to be expected as the anal papillae are the principal organs for the collection of salts, but it does not invalidate any of the arguments which have been developed.

The exchange occurring through the gut accounts for roughly 10% of the total exchange in both fed and starved larvae, so clearly feeding greatly increases the exchange through the gut. The very small exchange through the general body surface may be neglected (Treherne, 1954). Although some of the outflux through the gut no doubt occurs as a loss with the urine, it seems probable that most of the outflux occurs through the midgut at any rate in starved larvae, as blocking the mouth stops the medium entering the gut, and it greatly reduces the outflux from starved papilla-less larvae (Treherne, 1954). Arguments similar to the ones developed for the anal papillae may be applied to the gut, and it is possible that a similar sort of Na pump may occur in it. It is, however, not advisable to make too close a comparison between the two tissues at present as it is not yet known what proportion of outflux in papilla-less larvae occurs as losses with the urine and what effect feeding has on these losses. Furthermore, although the gut must be able to do osmotic work (as papilla-less larvae can keep the haemolymph sodium at about 80 mM/l. indefinitely despite any loss with the urine) it will be shown in a further paper that net transport inwards due to the gut occurs only in fed papilla-less sodium-deficient larvae which have been reared in distilled water. This net transport would appear to occur only after a considerable time lapse. However the gut has not been studied in much detail here and a more detailed study might well resolve these uncertainties.

Treherne's observations on the outflux of sodium from the haemolymph of starved larvae placed in flowing distilled water have been confirmed and extended. The outflux under these conditions is much smaller than it is when the larvae are in the medium. These results are compatible with the sort of sodium carrier and pump suggested earlier. It would be interesting to know how much of this outflux occurs through the anal papillae, the gut, the general body surface, and in the urine.

A considerable difference has been shown to exist between the anal papillae of fed and starved larvae. The exact significance of this difference is not at present apparent, but as there is good evidence that the chief function of the anal papillae is

to transport salts, it seems likely that the difference is correlated with the difference in the rates of exchange of sodium. For this reason the cytology of the anal papillae is worthy of further study, preferably by means of fixation with OsO_4 and electron microscopy.

In conclusion it should be pointed out that while for the sake of simplicity it has been assumed that the carrier mechanism occurs in some one barrier (in the anal papillae) between the haemolymph and the medium, the sodium has in fact to move through the internal and external cytoplasmic boundaries and through the cytoplasm itself. The implications of this sort of system are discussed in detail by Ussing (1948). The carrier models that have been suggested here are meant to describe only the overall fluxes of Na. It would be of interest to know the value of any potential differences between the haemolymph and the medium, and to test the effect upon the exchange of a wider range of sodium concentrations in the medium, and of a variety of metabolic inhibitors.

V. SUMMARY

1. The steady-state exchange of sodium in the 4th instar larva of *Aëdes aegypti* (L.) has been studied by means of flame photometry and ^{22}Na.

2. The steady-state exchange of sodium between the larva and medium is more rapid in fed larvae ($T_{\frac{1}{2}}$ about 10 hr.) than in starved larvae ($T_{\frac{1}{2}}$ about 60 hr.). There is no difference between the sodium levels of fed and starved larvae.

3. In both fed and starved larvae about 90 % of the exchange occurs through the anal papillae so these organs must be responsible for about 90 % of the difference between fed and starved larvae in the rate of exchange.

4. Cytological changes in the anal papillae following upon feeding and starvation are described.

5. The results are discussed in terms of possible carrier mechanisms in the anal papillae.

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THE JUMPING MECHANISM OF SALTICID SPIDERS

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(With Plates 13 and 14)

INTRODUCTION

There are no extensor muscles at the 'hinge joints' (femur-patella and tibia-basitarsus joints) of the spider leg. Parry & Brown (1959) have recently produced evidence that at these joints extension is due to the haemocoelic blood pressure in the leg: we measured this pressure, established an empirical relation between pressure and torque at the hinge joints, and used this relation to show that the observed pressures are adequate to account for the torques developed by the living spider.

In this paper we consider whether a hydraulic mechanism is likely to account for the jump of the jumping spider *Sitticus pubescens* (Fabricius: Salticidae). First a description of the jump is given and it is shown to depend almost entirely on the sudden straightening of the fourth pair of legs. From photographs of the jump we estimate the mean acceleration during take-off and hence the propulsive force and the torques at the hinge joints. Finally we use the relation between torque and blood pressure already established for the house spider *Tegenaria* sp. (Parry & Brown, 1959) to obtain a rough estimate of the blood pressures which would be needed to account for the jump.

DESCRIPTION OF THE JUMP

Method

The photographic equipment consists of a continuously-running camera synchronized with a British Thomson-Houston Type FA I discharge tube used as a multiple-flash light source (Brown & Popple, 1955). The spider is photographed while jumping across a gap of about 5 cm. from a narrow take-off platform to a wider landing platform. By idling the camera motor and then suddenly releasing the clutch when the spider is judged to be on the point of jumping, we were able to get a number of sequences of the jump taken at maximum camera speed—167 half-frames/sec. (6 msec. per half-frame) on 35 mm. film. In addition to these sequences we also took a number of multiple-image photographs of the complete jump, using a flash interval of 7 msec. and a 'black box' background. All this work was done at a temperature of about 20° C.

The jump

Unlike, for example, most jumping insects, the salticid spiders lack any obvious specialization of their legs and the way in which they jump does not seem to have

been determined. The jump of a mature male *Sitticus pubescens* is shown in Pl. 13, and the following points will be noted: (*a*) The fourth legs are held in a plane parallel to the vertical sagittal plane of the animal and straighten to their full length during the jump chiefly owing to extension at the femur-patella hinge joint and to depression of the femur; (*b*) the third legs are held laterally and move through a small angle before losing contact with the ground; (*c*) the first and second legs are held off the ground and do not contribute to the jump.

The multiple-image photographs (Pl. 14) show the use to which the drag-line silk is put during the jump. In Pl. 14*a* the animal executes a rather flat trajectory, drawing out a silk thread behind it (visible in the first two images and also bridging the gap at the end of the jump). Tension in this thread probably accounts for the reversal, in mid-air, of the animal's initial counter-clockwise rotation (pitch). In Pl. 14*b* there is a similar initial rotation, but here the tension in the thread appears to have suddenly increased so that not only is the rotation reversed but the animal drops almost vertically at the end of its jump. In Pl. 14*c* the thread seems to have broken and the animal makes a complete somersault.

It must be emphasized that the description just given of the salticid jump applies to *Sitticus pubescens* alone and that other species may jump differently, although we have no reason to believe that there are radical differences. For instance the zebra spider *Salticus scenicus* (Clerck) uses both the third and fourth pairs of legs when jumping, the third pair leaving the ground after the fourth pair. It is particularly fortunate for our purposes that in the species we happened to choose for study the jump is almost entirely due to a single pair of legs: otherwise the torques at the joints could not have been resolved.

TORQUES AT THE LEG JOINTS

Method

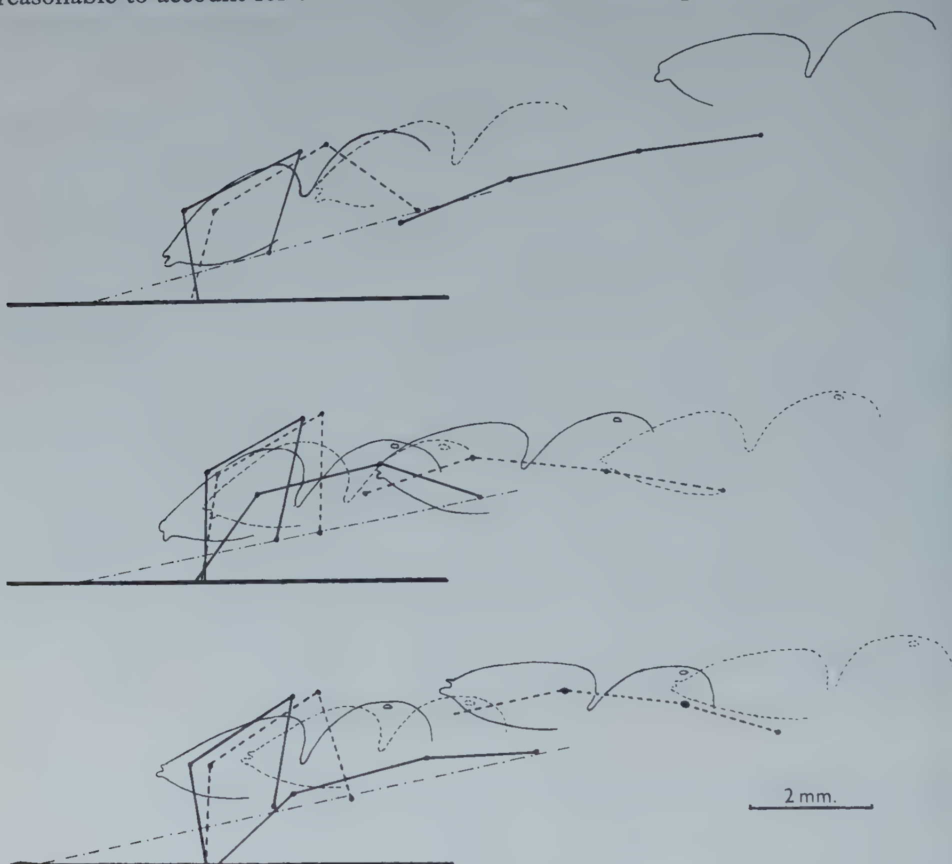
Details of the method used in calculating the torques at the leg joints during the jump are given in the appendix. In principle the method is as follows. From a photographic sequence we measure the angle of take-off, the distance moved by the spider's body during take-off, and the velocity of take-off. Thus we obtain the mean acceleration and, knowing the spider's mass, the magnitude and direction of the force acting on the spider. Taking this force to be equally distributed between the feet of the fourth pair of legs, we then calculate the torques at the joints for each of the leg configurations shown in the film.

The principal assumptions made in this calculation are:

(*a*) The third pair of legs contribute a negligible amount to the jump (the first and second legs need not be considered because they are held off the ground). This assumption is supported by the fact that removal of the third pair of legs has little effect on the take-off velocity of the jump—see 'other measurements of take-off velocity' below.

(*b*) The animal accelerates uniformly in a straight line. That the take-off path is roughly straight may be seen from the tracings of three jumps shown in Text-fig. 1. Whether the acceleration is uniform cannot be determined without a greatly

increased flash frequency and camera speed. This is one reason why our numerical results must be taken as approximate. They should, however, be adequate to show whether active extension is occurring at the hinge joints, and whether it would be reasonable to account for this extension in terms of blood pressure.



Text-fig. 1. Tracings of three jumps. The disposition of the fourth leg is shown as far as the trochanter-femur joint.

Results

Text-fig. 2 contains an analysis of eight jumps of two different spiders, each diagram showing the disposition of the near fourth leg in the two or three frames (6 msec. intervals) occupied by the jump. In each case the first leg position is the one found immediately before movement begins. The torques present at each hinge joint and at the trochanter-femur joint are shown on the diagrams (unbracketed bold figures), the units being dyne-cm. The following convention is observed in regard to sign: torques *not underlined* act clockwise on the more distal segment; torques *underlined* act anti-clockwise on the more distal segment. It will be seen that the torques at the two hinge joints are always such as to produce extension at these joints; whereas those acting on the femur tend to produce elevation at first

but may change to a depression towards the end of the jump. Except when the legs are almost outstretched the torques at the hinge joints are considerably larger than those at the base of the leg, the latter always being relatively small.

PRESSURE IN THE LEG

We have shown (Parry & Brown, 1959) that under static conditions the following relation between torque (C) and pressure (P) holds good for the *Tegenaria* hinge joint:

$$C = k_\theta \times P,$$

where k_θ is a constant for any given joint angle. If C is measured in dyne-cm. and P is measured in dynes/cm.^{2} then k_θ has the dimension cm.^{3} and is numerically equal to $\Delta v/\Delta\theta$ or the rate of change of joint volume with angle. It thus depends only on the geometry of the joint and for similar joints—such as the *Tegenaria* and salticid hinge joints—will vary with the cube of the linear dimension. Table 1 shows, for each hinge joint, first the values of k_θ for *Tegenaria* (Parry & Brown, 1959), and then the calculated values of k_θ for the two salticids used in our experiments, obtained by multiplying the *Tegenaria* values by the ratio of the cubes of the respective hinge lengths. The actual lengths of the hinges were as follows. Upper joint: *Tegenaria*: 0·830 mm.; salticid A: 0·247 mm.; salticid B: 0·304 mm. Lower joint: *Tegenaria*: 0·600 mm.; salticid A: 0·160 mm.; salticid B: 0·198 mm.

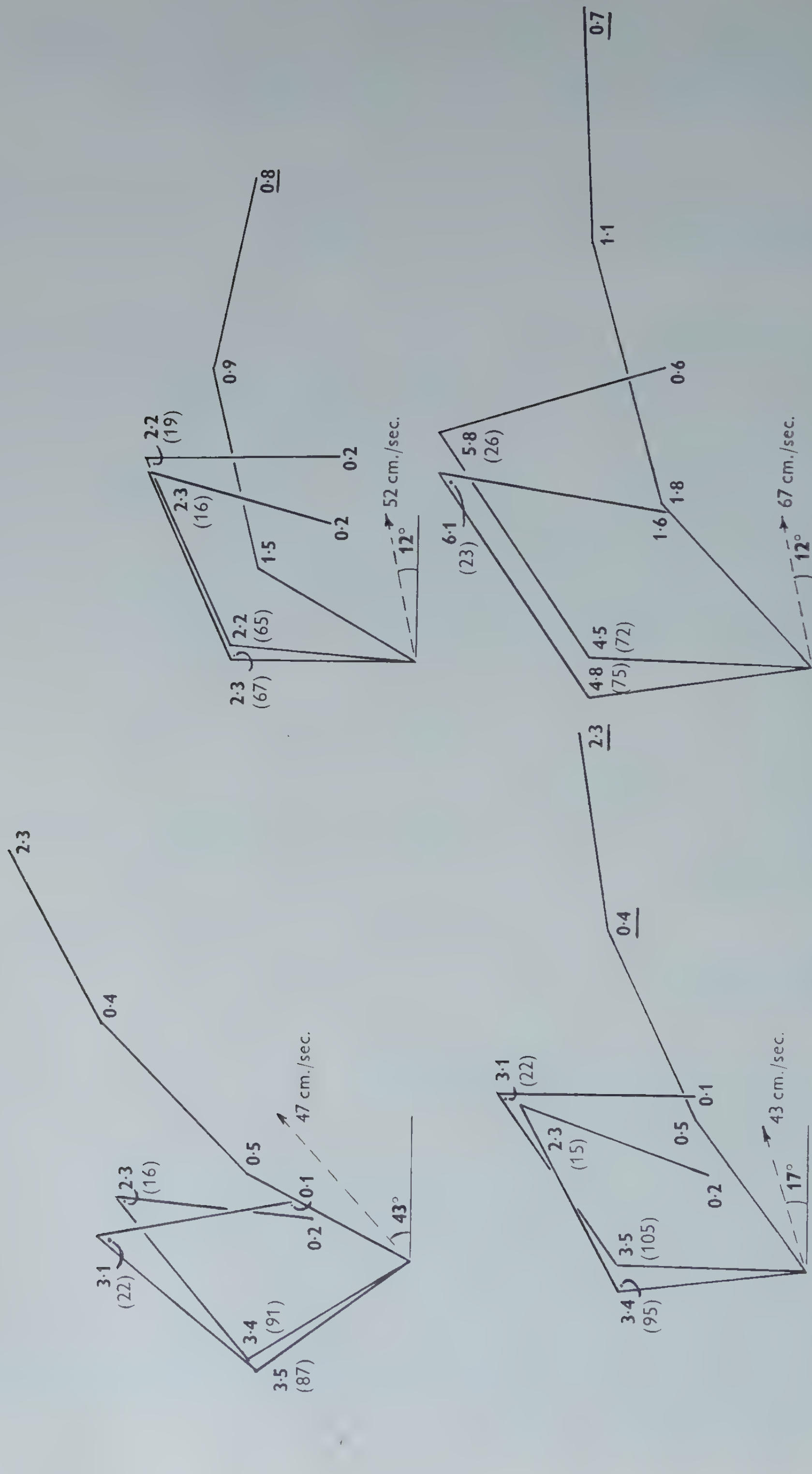
Table 1

Joint angle	40°	60°	80°	100°	120°	140°	160°
			Upper joint				
Tegenaria	440	370	320	310	300	290	200×10^{-3} mm.^{3} = k_θ
Sitticus, spider *A*	11	10	8	8	8	8	5
Sitticus, spider *B*	22	18	16	15	15	14	10
			Lower joint				
Tegenaria	—	—	170	150	130	130	120×10^{-3} mm.^{3} = k_θ
Sitticus, spider *A*	—	—	3	3	3	3	2
Sitticus, spider *B*	—	—	6	5	5	5	4

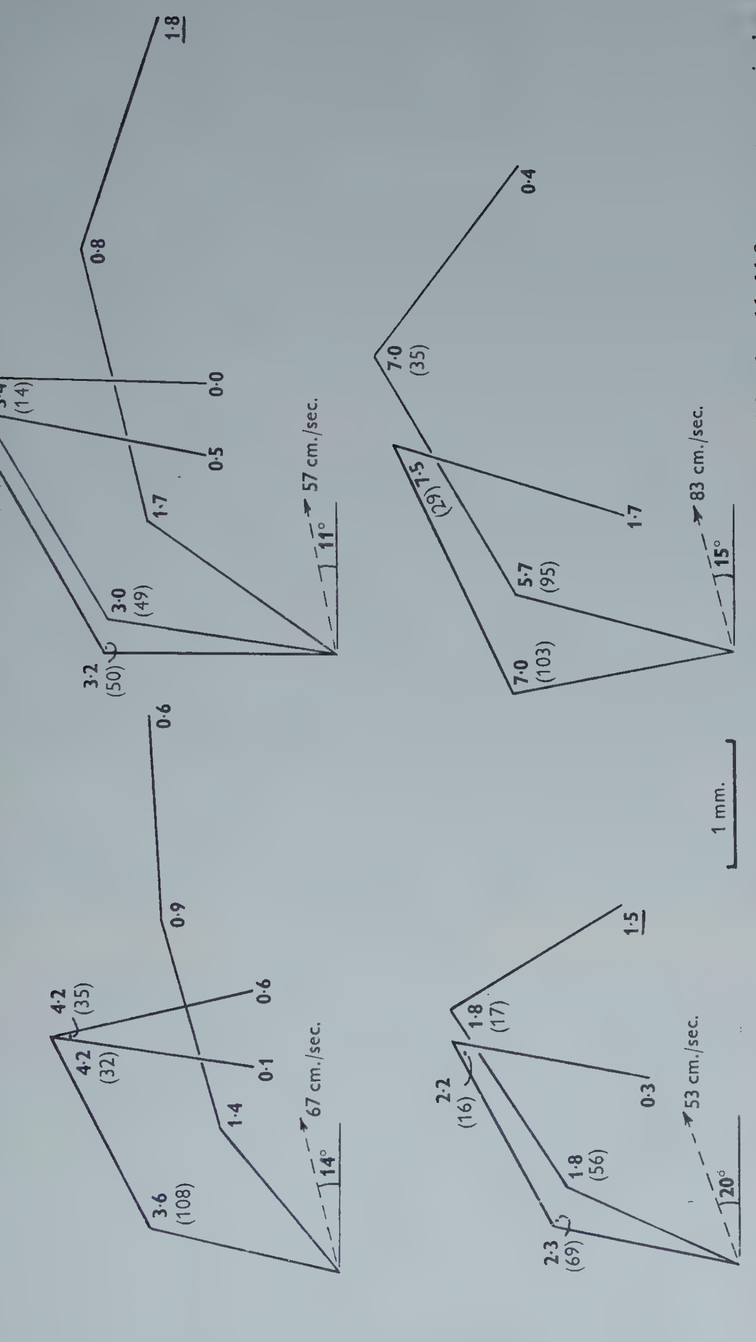
We have used these calculated values of k_θ to estimate the pressures needed to produce the torques developed during the jump—the pressure in cm. Hg is shown in parentheses in Text-fig. 2 beside the corresponding torques. It will be noted that:

(*a*) Although the torques are about the same at the two hinge joints, the corresponding pressures appear to be greater at the lower joint owing to its smaller size. In fact, unless viscosity effects are important, the pressure must be the same throughout the leg and the apparently lower pressure at the upper joint must therefore be due to flexor muscle tension.

(*b*) The absolute values of the calculated pressures can only be expected to indicate an order of magnitude because they are based on estimates of the torques and on the cube of a ratio of dimensions. Thus an error of 10% in the ratio of the



Text-fig. 2. For legend see p. 659.



Text-fig. 2. Tracings of the fourth leg up to the trochanter-femur joint during eight jumps. Unbracketed bold figures are torques in dyne-cm. (underlined figures indicate that the torque acts anti-clockwise on the more distal segment). Figures in parentheses are calculated pressures in cm. Hg. These are not shown when k_θ is small and changing rapidly—i.e. when the joint is becoming fully extended.

hinge lengths would produce an error of 25 % in the calculated pressures. In fact some of the pressures at the lower hinge joint are improbably high, but all lie within a factor of two and a half of the peak pressures actually measured in the leg of *Tegenaria* (Parry & Brown, 1959). Our results are therefore compatible with the idea that the hinge-joint torques are due to hydraulic pressure. It should be noted that, between similar animals of different size, the pressures will be similar because the force of a muscle is proportional to its cross-section—i.e. Force/Area ($\propto$ pressure) is constant.

Direct evidence that the blood pressure increases during jumping is provided by the behaviour of the leg spines which, in many spiders, become erect when pressure is applied to the body or to an isolated leg. Pl. 13 shows the spines erecting on the legs during the jump—this is clearly seen on the first and fourth legs and is also visible on the third legs (compare the first three sequences). The fact that erection is not limited to the jumping legs strongly supports the idea that the increase in pressure occurs throughout the prosoma.

OTHER MEASUREMENTS OF TAKE-OFF VELOCITY

Two other methods are available for measuring the take-off velocity and angle of take-off. These do not give the take-off distance and so the acceleration and force cannot be calculated; they do, however, provide an independent indication of the spiders' normal performance. These methods are:

(*a*) The spider is allowed to jump across a parallel beam of light so that its shadow falls on a sheet of graph paper. One observer notes the maximum height of the trajectory of the shadow while another observer notes the range and the height of the landing point above or below the take-off point. These measurements define the trajectory and enable the velocity of take-off and the angle of elevation to be calculated.

(*b*) From multiple-image photographs (e.g. Pl. 14) the angle of take-off and the maximum height can be measured: these parameters determine the velocity.

Estimations of the take-off velocity obtained by these two methods are shown in Table 2.

Table 2

	No. of observations	Mean velocity (cm./sec.)
Method (*a*)		
Spider *A*	25	79 ± 8
Spider *A* with 3rd legs removed	8	64 ± 6
Spider *B*	13	76 ± 9
Method (*b*)		
Spider *A*	12	75 ± 8

DISCUSSION

We have shown that when jumping *Sitticus pubescens* suddenly extends its fourth pair of legs, which contribute almost all the motive force. Two of the joints involved are hinge joints and lack extensor muscles, and yet we estimate that the extension

torques at these joints are considerable. Perhaps the most significant evidence that extension at the hinge joints is due to hydraulic forces comes from the unexpected discovery that the leg spines become erect during the jump—a result of increased body pressure which can be demonstrated on many spiders. Calculation of the actual pressures at the joints needed to produce the estimated torques can only be very approximate. They do, however, fall within a factor of about two of the peak pressures actually measured in the *Tegenaria* leg (Parry & Brown, 1959).

Although we have thus produced evidence that the jump is due to hydraulic forces, we are still far from understanding the detailed mechanism involved. Some of the main points yet to be decided are:

(*a*) Which muscles control the blood pressure? We believe they are in the prosoma—see Parry & Brown (1959) and also note that spine erection is not limited to the jumping legs.

(*b*) Do the muscles shorten during the jump; or do they do so before the jump begins, the work being stored as elastic energy?

(*c*) What pressure gradients occur within the leg due to the flow of blood into the expanding hinge joints?

We hope that further studies of larger spiders may throw more light on some of these questions.

The range of a projectile depends on the angle of projection and the square of the initial ('take-off') velocity $\{R = \sin 2\theta \times (v^2/g)\}$ and for a given terminal velocity is greatest when $\theta = 45°$. Usually *Sitticus pubescens* takes off at an angle less than 45° and in most of our determinations of take-off velocity it landed on a vertical surface placed up to 7 cm. away. The greatest take-off velocity encountered was 99 cm./sec., and if the angle had been 45° this would have produced a jump of 10 cm. provided there was no check from the drag-line. Energy considerations show that, *other things being equal*, range should be independent of size:

Range $\propto v^2$ (see above).

Work done by muscles $\simeq \frac{1}{2}mv^2$ (neglecting increase in potential energy during take-off which is relatively small).

$$\text{Therefore range} \propto \frac{\text{work done by muscles}}{\text{mass of spider}}.$$

Temperature is likely to be one of the most important factors influencing the work done by a given amount of muscle. Buchthal, Weis-Foch & Rosenfalck (1957) found that the twitch work of locust flight muscle increased by a factor of $\frac{5}{3}$ for an increase in 12° C. (20–32°). Thus temperature could account for jumps of 20 cm. performed by a South American salticid at an air temperature of 30° (we are grateful to Dr H. W. Lissmann for these observations). It might also account for jumps of 25 cm. reported by Pickard-Cambridge (1879) for *Attulus* ('*Attus*') *saltator* which lives on sandhills and in similar unsheltered environments which may get very hot on a sunny day.

The performance of salticid spiders in general compares very unfavourably with that of such jumping insects as the Acriidae. The jump of the latter is directly due

to the contraction of muscles which, owing to the gearing provided by the anatomy of the leg, may work at close to their optimal mechanical efficiency. In a hydraulic system the work which the muscles can do in a single contraction depends on the compliance (relation between dimensional changes and pressure) of the animal's body and on the volume change occurring at the leg joints. It appears that muscles operating such a system do so at considerably less than their optimum efficiency. However salticid spiders are small, so that although their absolute range is poor the range measured in terms of the body length is considerable, and this often has the greater biological significance.

SUMMARY

1. Photographs of the jumping spider *Sitticus pubescens* (Salticidae) show that the jump is almost entirely due to the sudden straightening of the fourth pair of legs. Multiple-image photographs show the importance of a silk drag-line in controlling the jump.

2. The torques at the leg joints have been estimated. Extension torques occur at the two hinge joints although these lack extensor muscles.

3. The erection of leg spines at the moment of the jump provides direct evidence that hydraulic forces are involved in the jump. This view is supported by estimates of the pressures involved, which fall within a factor of about two of those previously measured in the legs of the spider *Tegenaria*.

We are greatly indebted to Dr K. E. Machin for his advice on the analysis of the forc es involved in the ump, and for reading the manuscript.

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APPENDIX

This appendix shows in detail the method of calculating the torques at the joints of the salticid during a jump.

(*a*) *Calculation of the force acting at the foot* (see Text-fig. 3)

Let:

Take-off velocity $= v$ cm./sec. (measured on film).

Take-off distance $= s$ cm. (measured on film).

Mean acceleration $= \frac{1}{2}(v^2/s)$.

Mass of spider $= m$ g.

Mean resultant force through centre of gravity ($=$ mass $\times$ acceleration) $=$

$$\frac{v^2 m}{2s} = \bar{F}.$$

From a resolution of forces:

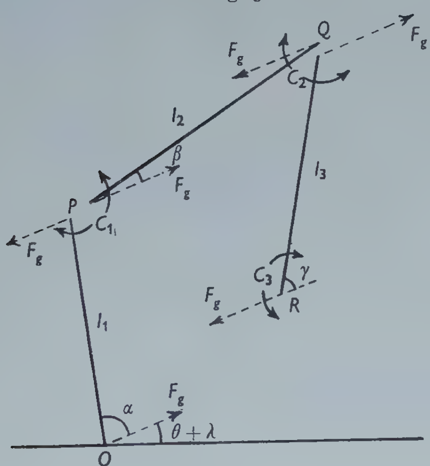
$$\text{Ground reaction } (F_g) = \frac{mg\cos\theta}{\sin\lambda}. \tag{Ia}$$

$$\tan\lambda = \frac{mg\cos\theta}{\bar{F}-mg\sin\theta}. \tag{Ib}$$

(b) Calculation of torques at the joints

In Text-fig. 4 let α, β and γ define the configuration of the leg, and l_1, l_2 and l_3 be the lengths of the segments OP, PQ and QR respectively. Also let C_1, C_2 and C_3 be the magnitudes of the equal and opposite torques at the joints P, Q and R.

Text-fig. 3



Text-fig. 4

Since the mass of a leg segment is small compared with that of the spider, it can be shown that changes of momentum and angular momentum can be neglected. Hence the sum of all the forces acting on a segment must be zero—i.e. the ground reaction F_g acting at the foot O will produce equal and opposite forces F_g at P, Q and R as shown (Text-fig. 4). Furthermore the sum of all the couples acting on a segment must also be zero. Thus:

(i) Segment OP (actually the two tarsal segments taken together) is acted upon by the anti-clockwise couple $F_g l_1 \sin\alpha$ and the couple C_1. Calling clockwise couples positive:

$$-F_g l_1 \sin\alpha + C_1 = 0.$$

$$\therefore C_1 = F_g l_1 \sin\alpha. \tag{1}$$

(ii) Similarly segment PQ (actually tibia and patella taken together) is acted upon by the anti-clockwise couple $F_g l_2 \sin\beta$ and the couples $-C_1$ and C_2, and

$$-F_g l_2 \sin\beta - C_1 + C_2 = 0.$$

Using (1):
$$C_2 = F_g(l_1 \sin\alpha + l_2 \sin\beta). \quad (2)$$

(iii) Similarly segment QR (the femur) is acted upon by the clockwise couple $F_g l_3 \sin\gamma$ and the couples $-C_2$ and C_3; and

$$F_g l_3 \sin\gamma - C_2 + C_3 = 0.$$

Using (2):
$$C_3 = F_g(l_1 \sin\alpha + l_2 \sin\beta - l_3 \sin\gamma). \quad (3)$$

(c) *A worked example*

The following calculation applies to the jump illustrated in Pl. 13:

Take-off velocity (v) = 67 cm./sec.
Take-off distance (s) = 0·44 cm.
Mean acceleration = 5,130 cm./sec.^{2}.
Mass of spider = 0·01 g.
Resultant force through centre of gravity ($\bar{F}$) = 51·3 dynes.
Angle of take-off (θ) = 12°.
Ground reaction (F_g) = 55 dynes (27·5 dynes per leg).
$\lambda = 10°$.

Dimensions of leg segments: $l_1 = 0{\cdot}18$ cm.; $l_2 = 0{\cdot}22$ cm.; $l_3 = 0{\cdot}19$ cm. Considering the disposition of the leg at the moment of take-off (Pl. 13, 2nd frame): $\alpha = 76°$; $\beta = 12°$; $\gamma = 58°$.

Hence from equations (1), (2) and (3):

$C_1 = 4{\cdot}8$ dyne-cm.; $C_2 = 6{\cdot}1$ dyne-cm.; $C_3 = 1{\cdot}6$ dyne-cm.

Similarly for the disposition of the leg shown in Pl. 13, 3rd frame:

$\alpha = 66°$; $\beta = 12°$; $\gamma = 84°$.
$\therefore C_1 = 4{\cdot}5$ dyne-cm.; $C_2 = 5{\cdot}8$ dyne-cm.; $C_3 = 0{\cdot}6$ dyne-cm.

Finally for the disposition of the leg shown in Pl. 13, 4th frame:

$\alpha = 21°$; $\beta = 7°$; $\gamma = 20°$.
$\therefore C_1 = 1{\cdot}8$ dyne-cm.; $C_2 = 1{\cdot}1$ dyne-cm.; $C_3 = -0{\cdot}7$ dyne-cm.

Note. The signs of C_1, C_2 and C_3 in this worked example indicate the sense of the couples acting at each joint on the *distal* segment.

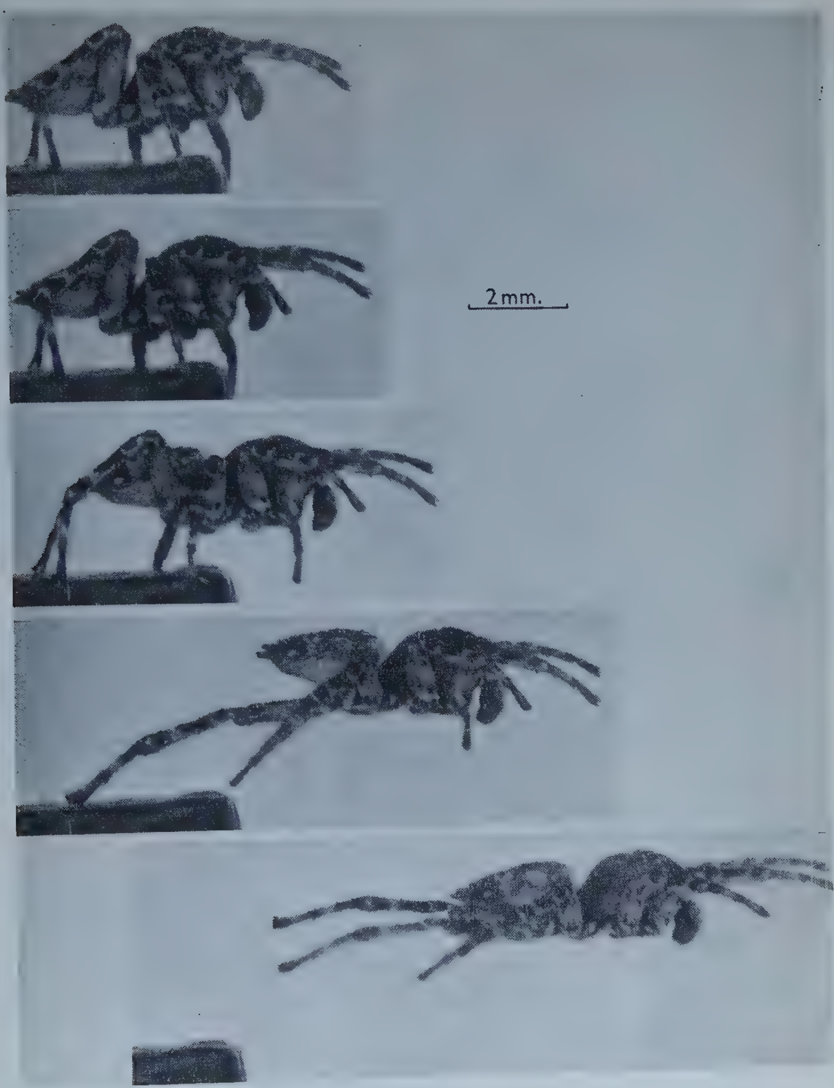
EXPLANATION OF PLATES

Plate 13

Sitticus pubescens jumping. Frame interval 6 msec. Note the inflation of the articular membrane of the femur-patella joint, and the erection of the leg spines.

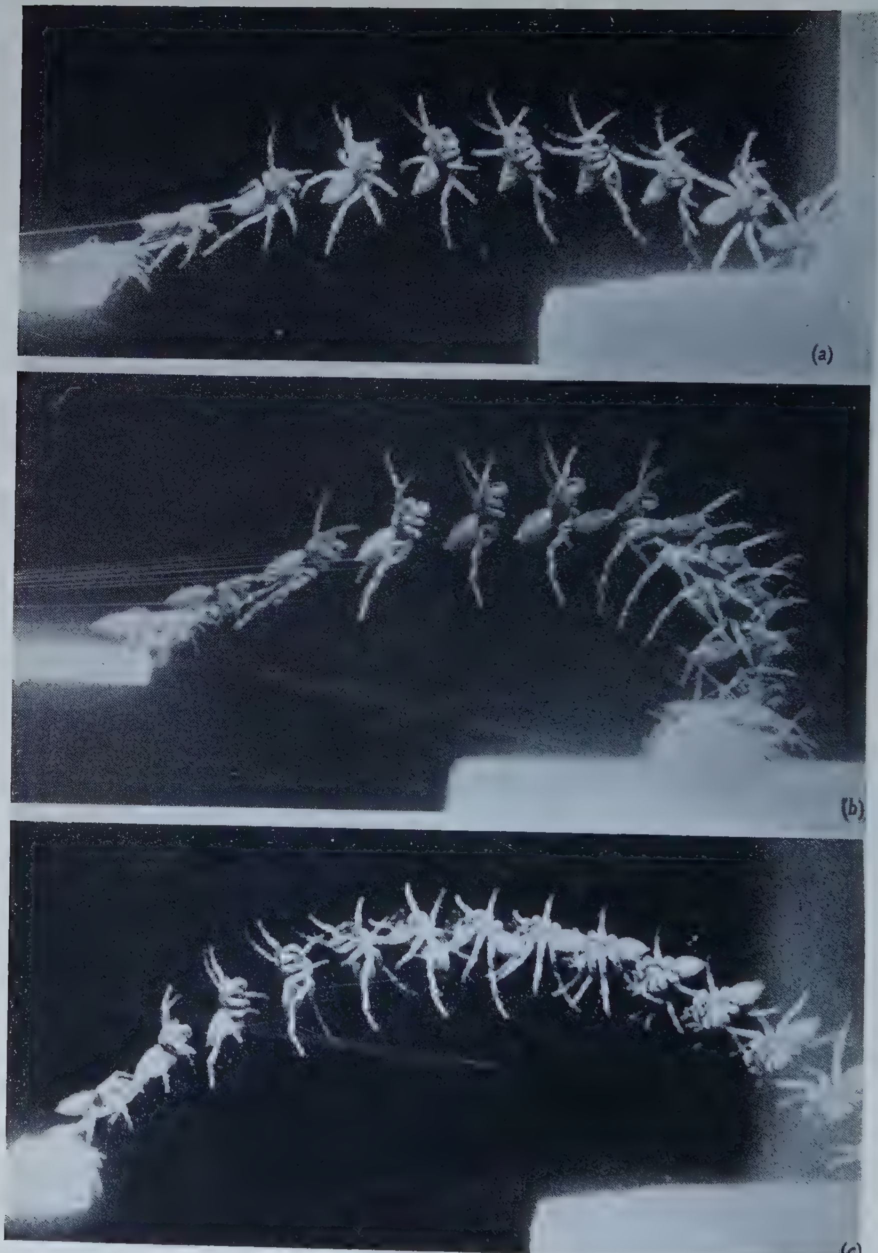
Plate 14

Sitticus pubescens jumping, taken by multiple-flash photography (flash interval $7\frac{1}{2}$ msec.). In *a* the silk drag-line appears to be used to check counter-clockwise pitch; in *b* it is used to control range; while in *c* it appears to have broken and the spider makes a somersault.



PARRY AND BROWN—THE JUMPING MECHANISM OF SALTICID SPIDERS

(*Facing p.* 664)



PARRY AND BROWN—THE JUMPING MECHANISM OF SALTICID SPIDERS

STUDIES ON THE METABOLISM OF LOCUST FAT BODY

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The successive accumulation and depletion of reserves in the fat body of insects shows that it is an important storage organ, but it seems possible that it is also an organ of intermediary metabolism, degrading some substances and elaborating others for use by other tissues. A study was made *in vitro* of the incorporation of certain metabolites, glycine, leucine, acetate and glucose, labelled with carbon-14, into the fat body of the desert locust, *Schistocerca gregaria* Forskål. This showed something of the degradation and synthesis occurring in locust fat body and also suggested that the tricarboxylic acid cycle functioned normally, contrary to an earlier report (Hearfield & Kilby, 1958). The results obtained from these studies on the incorporation of labelled metabolites and on the respiration of locust fat body are presented here.

The fat body of *S. gregaria* becomes packed with fat droplets, the predominant reserve. In addition it contains abundant glycogen and protein, and ribonucleic acid can be readily demonstrated in the cytoplasm; the tissue is free from symbiotic organisms (Coupland, 1957). *Schistocerca* fat body has been shown to contain a number of enzymes including transaminases and the glutamic dehydrogenase system (Kilby & Neville, 1957) and an enzyme system which synthesizes trehalose from glucose (Candy & Kilby, 1959).

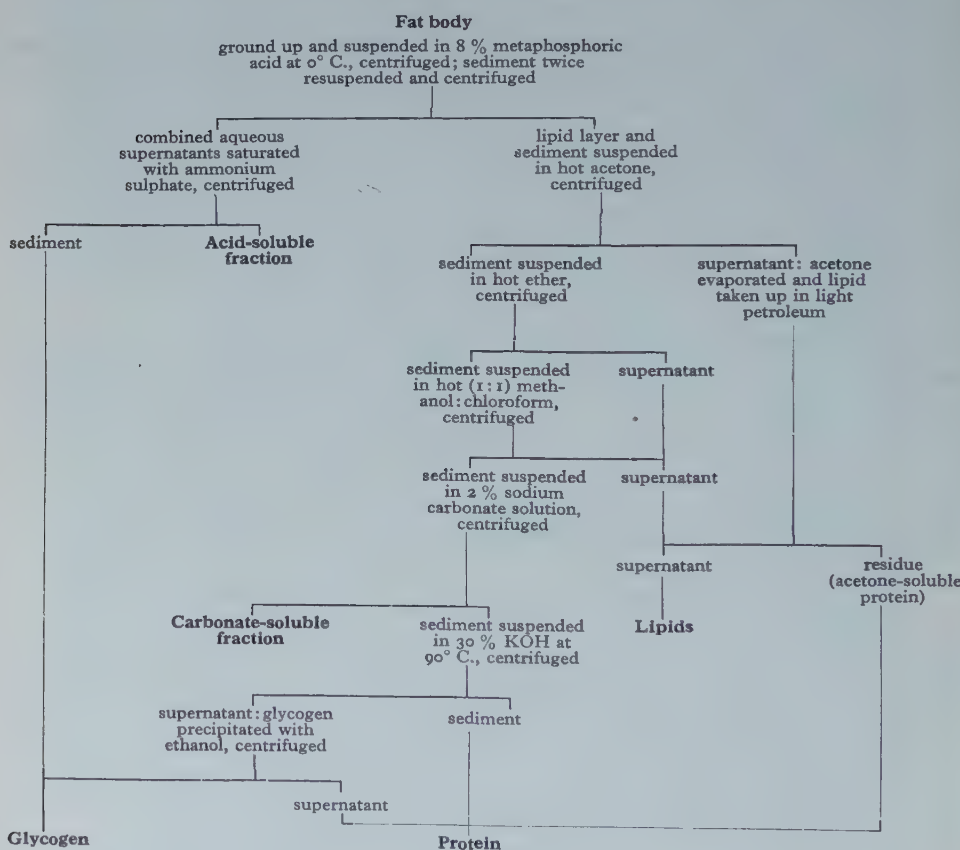
MATERIAL AND METHODS

Experimental animals. S. gregaria Forskål was chosen because its fat body could be removed in a sheet which remained intact throughout experiments. The fat body was taken from sexually immature males of the gregarious phase approximately 5 days after the moult to the adult stage when it was actively laying down reserves. In the few experiments where locusts of a different age were used this is mentioned in the text. The locusts were obtained from the Anti-Locust Research Centre a day or two before use and were fed.

Incubation procedure. Locusts were anaesthetized and opened in a saline devised for *Schistocerca* (Weis-Fogh, 1956; note erratum). The perivisceral fat body, which contains fewer oenocytes than the peripheral fat body (Coupland, 1957), was removed from the insect and freed from testes and air sacs. The sheets of fat body were incubated separately in manometer flasks containing locust saline and trace amounts (approximately 0·5 μc.) of a metabolite labelled with carbon-14.

Incubation lasted for 4 hr., the flasks being kept at 30° C. and shaken, and the carbon dioxide produced was collected on slips of filter paper moistened with potassium hydroxide solution which had been placed in the centre wells.

Fractionation procedure. After incubation the fat body was carefully rinsed and then divided into the following fractions: acid-soluble, glycogen, fat, carbonate-soluble and protein. After addition of carrier glycogen, the tissue was ground with extra-fine carborundum powder in the presence of freshly-made 8 % metaphosphoric acid at 0° C. A flow diagram of the fractionation procedure is shown below. The procedure was designed to remove both water-soluble and protein-bound glycogen. Extraction of lipids was carried out at 45–50° C. and the acetone-soluble protein was separated from the lipids by its insolubility in light petroleum.



The extraction with sodium carbonate solution was included to remove uric acid which is known to be present in fat body. Nucleic acids also dissolve in this solution. It was subsequently found that the small amount of radioactivity in the carbonate-soluble fraction (1–4 %) was not in uric acid or nucleic acids. A tentative identification of labelled glycine in a hydrolysate of the fraction, after incuba-

tion with glycine-^{14}C, suggested that some protein had been taken up in solution.

Preparation of cell-free tissue suspensions. Sheets of perivisceral fat body were dissected from locusts as described above, rinsed in 0·15 M potassium chloride and homogenized by hand in a glass homogenizer at 0° C. The homogenate was suspended in the potassium chloride solution and centrifuged twice at 500 g. to remove cellular debris, nuclei and fat. The contents of each manometer flask consisted of a cell-free suspension of fat body, equivalent to the sheet normally removed from one locust, in 0·15 M potassium chloride, pH 7·4, and 0·5 μc. succinic acid-1,4-^{14}C. Half of the flasks also contained 0·01 M sodium malonate and in one experiment 0·001 M ethylene-diamine-tetra-acetic acid was added to each flask. Incubation proceeded for 1 hr. at 30° C. in a gas phase of air, the flasks being shaken. Carbon dioxide produced during the experiment was released from solution by addition of acid and collected on filter paper moistened with potassium hydroxide solution previously placed in the centre well.

A sheep heart succinic oxidase system was prepared as described by Umbreit, Burris & Stauffer (1957). A second preparation was made in which the sheep heart muscle was homogenized with half its weight of locust fat body and the homogenate allowed to stand for an hour at 0° C. before removal of the fat by centrifuging. The final incubation mixture consisted of the homogenate in 0·1 M phosphate buffer, pH 7·4, and 0·05 M sodium succinate. The homogenates were incubated with shaking at 37° C. in an atmosphere of air. Oxygen uptake was expressed as Q_{O_2}, i.e. μl. oxygen/mg. fat free dry wt./hr.

Analytical methods. The radioactivity of the fat-body fractions was measured by gas analysis after oxidation of tissue carbon to carbon dioxide (Glascock, 1954). The radioactive compounds of the acid-soluble fraction were separated on paper chromatograms and located by the automatic scanning technique of Winteringham, Harrison & Bridges (1952). The radioactive spots were eluted and run again (Winteringham, 1953) and were identified by co-chromatography with known compounds in at least three solvent systems, those principally used being (i) water-saturated phenol; (ii) butanol:ethanol:water (40:10:50), and (iii) formic acid: acetone:water (14:60:26). The radioactive amino acids in the protein fraction were identified in the same way after hydrolysis of the protein. Lipids were analysed on silicic acid columns (Borgström, 1952*a*, *b*).

THE INCORPORATION OF METABOLITES LABELLED WITH CARBON-14 INTO *SCHISTOCERCA* FAT BODY

Sheets of perivisceral fat body from immature adult male locusts were incubated for 4 hr. at 30° C. in locust saline with trace amounts of metabolites labelled with carbon-14. The carbon dioxide produced during incubation was collected in the usual way. After incubation the fat body was rinsed and fractionated. The distribution of radioactivity in the various fractions and the radioactivity of the carbon dioxide, after incubation with various metabolites, is shown in Table 1.

Table 1. *Distribution of radioactivity in fat-body fractions after incubation of whole tissue with* ^{14}C*-labelled metabolites for 4 hr.*

Stage	Metabolite	No.	% incorporation into fat body fractions (mean ± S.E.)					Radioactivity of CO_2 as % of total uptake, i.e. fat body + CO_2 activity
			Acid-soluble	Glycogen	Fat	Carbonate-soluble	Protein	
Immature adult ♂♂	Glycine-^{14}C (G)	8	33·3 ± 3·2	2·8 ± 0·6	23·5 ± 2·7	4·5 ± 0·4	35·9 ± 4·5	63·8 ± 2·4
5th instar larvae ♂♂	Glycine-^{14}C (G)	6	33·2 ± 1·7	3·2 ± 0·4	25·5 ± 3·9	3·6 ± 0·3	34·4 ± 3·8	69·4 ± 6·3
Immature adult ♂♂	Leucine-^{14}C (G)	8	30·3 ± 2·0	1·1 ± 0·1	14·9 ± 5·0	2·2 ± 0·6	51·5 ± 3·5	55·8 ± 7·5
Immature adult ♂♂	Acetate-2-^{14}C (G)	8	21·0 ± 4·1	1·0 ± 0·1	55·0 ± 6·3	2·5 ± 0·4	20·5 ± 3·2	35·7 ± 4·5
Immature adult ♂♂	Glucose-^{14}C (G)	8	59·6 ± 5·6	3·0 ± 0·6	22·8 ± 4·3	4·1 ± 0·6	10·4 ± 1·7	30·0 ± 3·2

Glycine-^{14}C (*G*). In the fat body of immature adults one-third of the radioactivity was in the acid-soluble fraction. The radioactivity of the glycogen and of the carbonate-soluble fraction was very low. Fat radioactivity was fairly high (23 %) as was that of the protein (35 %). The radioactivity of the carbon dioxide was higher than that of all the fat body fractions combined, showing that the tissue oxidized more glycine than it incorporated. To illustrate this point, carbon dioxide radioactivity is expressed, in Table 1, as percentage of the total uptake of carbon-14, i.e. as percentage of fat body plus carbon dioxide radioactivity.

The results obtained with fat body taken from male larvae 5 days after entering the 5th instar were almost identical with those from immature adults (Table 1) and preliminary results with sexually mature adult males showed that the pattern of incorporation had not changed.

The various fractions of fat body from the immature adult were analysed further. Radiochromatography of the acid-soluble fraction showed that 76 % of the radioactivity was in glycine and serine. The remainder of the activity was in two spots which were not identified. Hydrolysis of the protein fraction showed that the radioactivity was again in glycine and serine. Analysis of the fat fraction showed that 95 % of the radioactivity was in neutral fat. The remainder was distributed between free fatty acids, phospholipids, cholesterol and cholesterol esters. Hydrolysis of the neutral fat showed that 55 % of its activity was in fatty acid. Hydrolysis of the carbonate-soluble fraction yielded a radioactive compound tentatively identified as glycine.

Leucine-^{14}C (*G*). Incubation with generally-labelled leucine yielded a pattern of incorporation somewhat similar to that obtained with glycine. The principal differences were in lower fat and higher protein radioactivity. Once again the carbon dioxide was highly radioactive, containing more than half of the carbon-14 taken up by the fat body.

Sodium acetate-2-^{14}C. The radioactivity of the acid-soluble fraction was lower and that of the fat fraction higher than after incubation with the other metabolites. Glycogen radioactivity was again negligible and the protein showed moderate radioactivity. Only one-third of the carbon-14 taken up by the fat body was converted to carbon dioxide. The radioactivity of the acid-soluble fraction was principally in proline and glutamate, the proline usually being rather more radioactive than the glutamate. Aspartate was found to be radioactive on one occasion and radioactive trehalose was also found once. There was no evidence of labelled glutamine. The acid-soluble fraction gave clearer radiochromatograms after hydrolysis suggesting that labelled peptides were present. After hydrolysis of the protein fraction radioactivity was found in proline, glutamate, aspartate and alanine, the glutamate and aspartate being the most heavily labelled.

Glucose-^{14}C (*G*). The most striking feature of the incorporation of generally-labelled glucose into fat body was the high radioactivity of the acid-soluble fraction which comprised nearly 60 % of the fat-body activity. Radiochromatography showed that the activity was almost entirely confined to trehalose. Only 3 % of the glucose was incorporated into glycogen; 22 % was incorporated into fat corre-

sponding to the result obtained with glycine; incorporation into protein was low. Hydrolysis of the carbonate-soluble fraction yielded a single radioactive compound. This was not identified but it was found not to be glucose, ribose, alanine or glutamate. Less than one-third of the carbon-14 taken up by the fat body was converted to carbon dioxide.

EXPERIMENTS ON THE RESPIRATORY METABOLISM OF *SCHISTOCERCA* FAT BODY

Hearfield & Kilby (1958) made an intensive study of oxidative enzymes in the fat body of *Schistocerca gregaria*. They showed that a number of enzymes associated with the tricarboxylic acid cycle were present, namely, aconitase, *iso*-citric dehydrogenase, fumarase, malic dehydrogenase and cytochrome oxidase; but they were unable to show the presence of the condensing enzyme, α-ketoglutarate oxidase or succinic dehydrogenase and for this reason suggested that the tricarboxylic acid cycle might not play an important part in the intermediary metabolism of locust fat body.

Succinic dehydrogenase has been demonstrated within isolated mitochondria of the fat body of *Periplaneta americana* (L.) by the reduction of tetrazolium salts to formazan dyes (Pearse & Scarpelli, 1958). Using a similar technique, β-hydroxybutyric dehydrogenase was demonstrated in mitochondria isolated from the fat body of *Locusta migratoria* L. (Hess, Scarpelli & Pearse, 1958), but attempts to obtain a reaction for succinic dehydrogenase in these mitochondria were unsuccessful (A. G. E. Pearse, personal communication). Bellamy (1958), obtained a very small uptake of oxygen on incubating homogenates of *Schistocerca* fat body with α-ketoglutarate and succinate and he suggested that α-ketoglutarate oxidase and succinic dehydrogenase were present but were labile on homogenizing.

When *Schistocerca* fat body was incubated with sodium acetate-2-^{14}C the radioactivity of the acid-soluble fraction was found to be in glutamate, proline and aspartate, as described above. This suggested that the tricarboxylic acid cycle was functioning normally since glutamate is formed from α-ketoglutarate by transamination and aspartate is formed from oxaloacetate in the same manner. Proline can be formed from glutamate. These experiments were therefore extended to study the respiratory metabolism of the fat body.

The production of carbon dioxide from sodium acetate and succinic acid

In a series of experiments, sheets of fat body and cell-free suspensions of fat body were incubated with sodium acetate-2-^{14}C or succinic acid-1,4-^{14}C and the radioactivity of the carbon dioxide produced was taken as an indication of the oxidation of the substrate. The results are given in Table 2.

Incubation of whole fat body with sodium acetate-2-^{14}C in the presence of 0·015 M sodium fluoroacetate inhibited the production of $^{14}CO_2$ by 95 %. The inhibitory effect of fluoroacetate has been described by Peters (1955). Fluoroacetate itself has no known action upon isolated enzymes *in vitro*, but *in vivo* fluoroacetate is

synthesized by the condensing system to monofluorocitric acid and this competitively inhibits aconitase, blocking the tricarboxylic acid cycle at the citric acid stage. The inhibitory effect of fluoroacetate on $^{14}CO_2$ production by whole fat body thus provides support for Hearfield & Kilby's claim to have found aconitase and provides indirect evidence for the presence of the condensing system of enzymes in locust fat body (Table 2).

Table 2. *Production of $^{14}CO_2$ by fat-body preparations from metabolites labelled with carbon-14*

Tissue preparation	Metabolite	No. of preparations	Inhibitor	Duration of experiment (hr.)	Mean radioactivity of CO_2/mg. dry wt. of preparation (c./m.)
Fat-body sheet in saline	Acetate-2-^{14}C	7	0·015 M fluoroacetate	3	45
	Acetate-2-^{14}C	7	—	3	985
Fat-body sheet in saline	Acetate-2-^{14}C	8	0·01 M malonate	3	2247
	Acetate-2-^{14}C	8	—	3	1717
Fat-body sheet in saline	Succinic acid-1,4-^{14}C	4	0·01 M malonate	3	4170
	Succinic acid-1,4-^{14}C	4	—	3	3888
Fat-body sheet in saline	Succinic-acid-1,4-^{14}C	8	0·02 M malonate	3	3600
	Succinic acid-1,4-^{14}C	8	—	3	4498
Cell-free fat-body suspension in 0·15 M-KCl	Succinic acid-1,4-^{14}C	4	0·01 M malonate	1	173
	Succinic acid-1,4-^{14}C	4	—	1	424
Cell-free fat-body suspension in 0·15 M-KCl + 0·001 M-EDA	Succinic acid-1,4-^{14}C	4	0·01 M malonate	1	107
	Succinic acid-1,4-^{14}C	4	—	1	515

Incubation of whole fat body with succinic acid-1,4-^{14}C yielded highly radioactive carbon dioxide, showing that an active succinic dehydrogenase was present. To confirm this finding, the effect of malonate on $^{14}CO_2$ production was examined. 0·01 M malonate had no inhibitory effect on $^{14}CO_2$ production during incubation of whole fat body with trace amounts of acetate-2-^{14}C or succinic acid-1,4-^{14}C; in fact, in all experiments it appeared to have a slightly stimulating effect. Malonate is known to stimulate endogenous respiration in *Chlorella* (M. Merritt, personal communication). The presence of 0·02 M malonate during incubation of whole fat body with succinic acid-1,4-^{14}C caused 20% inhibition in the production of $^{14}CO_2$ and the presence of 0·01 M malonate during incubation of a cell-free fat-body suspension with this metabolite caused inhibition of 59 and 79% in two experiments.

The effect of homogenization on the succinic oxidase system

Homogenization of fat body greatly reduced formation of $^{14}CO_2$ on subsequent incubation with succinic acid-1,4-^{14}C. Addition of 0·001 M ethylene-diamine-

tetra-acetic acid to the preparation had no marked effect. The reduction was greater than the figures in Table 2 suggest, for there the radioactivity of the carbon dioxide is expressed in terms of the dry weight of the final preparations. In whole fat body the dry weight included the inactive reserves, but in the cell-free suspensions the fat reserves were removed after centrifuging. Homogenization in fact reduced $^{14}CO_2$ production by about 95 %.

Sodium oleate is known to inhibit succinic oxidase (Edwards & Ball, 1954) and rat intestinal mucosa has been shown to contain a factor, identified as fatty acid, which inhibits succinic oxidase after homogenization (Nakamura, Pichette, Broitman & Bezman, 1959). It seemed likely that such an inhibitor must be released during homogenization of fat body so the effect of fat body homogenate on a succinic oxidase system known to be active was studied. Sheep heart muscle homogenized with half its weight of locust fat body gave a Q_{O_2} of 257 on incubation with 0·05 M succinate compared with the Q_{O_2} of 241 of the control preparation. The fat-body homogenate thus had no inhibitory effect on the sheep heart muscle succinic oxidase system.

The production of carbon dioxide from amino acids

When whole fat body was incubated with glycine-^{14}C (G) or leucine-^{14}C (G) well over half of the carbon-14 taken up was converted to carbon dioxide yet incubation with acetate-2-^{14}C or glucose-^{14}C (G) resulted in much less than half of the carbon-14 taken up appearing in this form (Table 1). This suggested that amino acids might form an important substrate for respiration so a comparison was made of the oxidation of amino acids by the fat body and by the indirect flight muscles. These tissues were incubated in locust saline with trace amounts of glycine-^{14}C (G) and leucine-^{14}C (G) and the radioactivity of the carbon dioxide produced was measured and expressed in terms of the dry weight of the tissue. Bellamy (1958) has shown that *Schistocerca* fat body and flight muscle have similar rates of respiration when incubated in 0·25 M sucrose. The results are given in Table 3.

Table 3. *Production of* $^{14}CO_2$ *by whole fat body and flight muscle from glycine-*^{14}C (G) *and leucine-*^{14}C (G) *during incubation in saline for* 4 *hr.*

Tissue	Metabolite	No.	Mean radioactivity of CO_2/mg. dry wt. (c./m.)
Flight muscle	Glycine-^{14}C (G)	4	331
Fat-body sheet	Glycine-^{14}C (G)	4	2465
Flight muscle	Leucine-^{14}C (G)	4	330
Fat-body sheet	Leucine-^{14}C (G)	4	8795

The fat body produced seven times as much radioactive carbon dioxide from glycine-^{14}C (G) as the flight muscle and it produced twenty-six times as much radioactive carbon dioxide from leucine-^{14}C (G) as the flight muscle.

DISCUSSION

Studies of isotope incorporation can provide very useful data on the pathways of metabolism but the recycling of isotopes in living tissues is liable to give an impression of synthesis where in fact there is no net gain. Thus the incorporation of radioactive glycine into protein may not represent synthesis of protein, but may simply reflect the rate of turnover of protein in the tissue. However, turnover rates are much lower in growing cells. In yeast the protein breakdown in growing cells is only 4 % of that in resting cells (Halvorson, 1958), and in rapidly growing cells of *Escherichia coli* there is no detectable turnover of protein (Mandelstam, 1958). As the fat body of *Schistocerca* was studied at a time when it was rapidly laying down reserves it may well be that the pattern of incorporation reflected synthesis rather than turnover but this cannot be taken for granted.

The pattern of incorporation obtained with the various metabolites shows that fat body is able to use a wide range of substrates for the formation of fat and protein although, as would be expected, glycine and leucine were more readily incorporated into protein than the other metabolites and acetate was more readily incorporated into fat. Sugars seem to be the only important source of trehalose. The high level of incorporation of various metabolites into protein suggests that fat body may synthesize much protein. In contrast, glycogen appears to be a minor product of fat-body synthesis and the high incorporation of glucose into trehalose may reflect the importance of trehalose as a fat-body reserve. It is known to be used during flight in *Locusta* (Bücher & Klingenberg, 1958).

Incubation with acetate-2-^{14}C led to the appearance of radioactivity in glutamate, aspartate and alanine. It is well known that these amino acids can become labelled with acetate via the tricarboxylic acid cycle and the result shows that in insects, as in other organisms, the intermediates of the tricarboxylic acid cycle can provide the carbon skeletons of the non-essential amino acids. It is surprising that no radioactivity was found in glutamine as Bellamy (1958) found glutamine to be twice as abundant as glutamate in *Schistocerca* fat body.

The inhibitory effect of fluoroacetate on fat body respiration confirms the presence of aconitase, found by Hearfield & Kilby (1958), and provides indirect evidence for the presence of the condensing enzyme system, not found by these authors.

The production of $^{14}CO_2$ from succinic acid-1,4-^{14}C shows that whole fat body contains succinic dehydrogenase, and this enzyme could be inhibited with 0·02 M malonate. The succinic oxidase system appears to be labile on homogenization, as was suggested by Bellamy (1958). The failure of Hearfield & Kilby (1958) to find the condensing system suggests that it also is labile on homogenization and it is of interest that inhibition at this point in the tricarboxylic acid cycle would lead to accumulation of oxaloacetate which would competitively inhibit succinic dehydrogenase. That the inhibition of succinic dehydrogenase is not due only to oxaloacetate accumulation is shown by Pearse's failure to demonstrate the enzyme in isolated mitochondria using a tetrazolium salt technique (see p. 670). It may be

concluded that some unknown factor of the homogenate inhibits the fat body condensing system and succinic dehydrogenase and, probably, α-ketoglutarate oxidase also. This factor has no effect on sheep heart muscle succinic dehydrogenase.

The much higher rate of oxidation of amino acids than of glucose to carbon dioxide is surprising as glucose is the most generally usable fuel, but it may be related to the functioning of the fat body in the intermediary metabolism of the insect. The demonstration in *Schistocerca* fat body of active transaminases and of a glutamic dehydrogenase system which links nitrogen and carbohydrate metabolism (Kilby & Neville, 1957) lends support to this idea. The finding that fat body oxidizes glycine and leucine many times faster than do the flight muscles parallels observations on the rat which showed that liver and kidney were able to oxidize glycine at a high rate while skeletal muscle had no such ability (Nakada & Weinhouse, 1953). It is possible that the fat body performs one of the functions of the vertebrate liver, transdeaminating amino acids and making the residues available for metabolism by other tissues.

SUMMARY

1. The incorporation of glycine-^{14}C (G), leucine-^{14}C (G), sodium acetate-2-^{14}C and glucose-^{14}C (G) into *Schistocerca* fat body was studied under *in vitro* conditions, and the distribution of radioactivity in the various fat body fractions and the labelling of compounds within the fractions is described.

2. The overall picture was of high incorporation into fat and protein and of very low incorporation into glycogen.

3. Incubation with glycine-^{14}C led to radioactivity appearing in the glycine and serine of the protein and of the amino acid pool. Incubation with sodium acetate-2-^{14}C led to radioactivity appearing in glutamate, proline, aspartate and alanine, showing that the intermediates of the tricarboxylic acid cycle provide the carbon skeletons of certain amino acids. Glucose-^{14}C was largely converted to trehalose.

4. Succinic dehydrogenase and the condensing enzyme system were shown to be present in fat body, contrary to previous reports. The succinic oxidase system was highly labile on homogenizing the tissue.

5. Fat body, unlike flight muscle, used glycine-^{14}C and leucine-^{14}C as respiratory substrates, and it is suggested that fat body acts like the vertebrate liver by transdeaminating amino acids and making them available for further metabolism by other tissues.

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THE SODIUM FLUXES IN THE MUSCLE FIBRES OF A MARINE AND A FRESHWATER LAMELLIBRANCH

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INTRODUCTION

The body fluids of most animals are rich in sodium whilst the tissues are generally rich in potassium and poor in sodium. In those tissues which have been examined in detail, such as striated muscle, nerve or red blood cells, it has been found that the ionic contents of the cells are determined by the active extrusion of sodium and sometimes by the active accumulation of potassium, combined with a Donnan equilibrium of some of the ions. The active extrusion of sodium from a cell against an electrochemical potential requires energy. This energy can be calculated for any tissue from the electrochemical potentials of the sodium in the intracellular and extracellular fluids and from the rate of extrusion of sodium from the tissue.

The sodium concentrations in the blood of marine invertebrates are very much higher than in the blood of freshwater and terrestrial animals, and for this reason it seemed probable that the flux of sodium through the tissues of a marine invertebrate would be greater than the flux of sodium through the comparable tissues of a freshwater animal. The energy required to extrude the sodium from the cells might be considerably greater in a marine invertebrate than in a freshwater form as the energy required is a product of the flux of sodium and the electrochemical potentials of the sodium.

Tracer techniques have been used to measure the movement of sodium through amphibian and mammalian muscle (Keynes, 1954; Creese, 1954) and vertebrate and invertebrate nerve (Dainty & Krnjevic, 1955; Keynes, 1951, etc.), but no results are available for comparable tissues of a related marine and freshwater invertebrate. This paper records the results of experiments designed to measure the rate of exchange of sodium in the muscles of a marine lamellibranch, *Mytilus edulis*, in which the sodium concentration in the blood is similar to that of sea water, and of a freshwater lamellibranch, *Anodonta cygnaea*, in which the concentration of sodium in the blood is less than 3% of that in sea water.

MATERIALS

The rate of exchange of sodium between the cells and the blood was determined by equilibrating a piece of muscle with a saline containing ^{24}Na and then measuring the declining activity of the sodium in the muscle when the muscle was exposed to

a current of tracer-free saline. In order to determine the rate constant of the exchange of sodium in the intracellular phase it is necessary that the loss of ^{24}Na by diffusion from the extracellular phase should be very rapid, otherwise some of the labelled sodium leaving the cells may re-enter other cells instead of escaping from the tissue. The slower the rate of loss from the extracellular fraction the greater the error from this cause. If the rate of loss from the extracellular phase is too low it becomes impossible to distinguish between the intracellular and the extracellular phases.

A suitable tissue for the experiment must have two properties. It must be in the form of a thin sheet so that the rate of loss from the extracellular phase will be rapid, and it must survive well *in vitro* so that the ionic composition at the end of the experiment is similar to that of the fresh tissue, otherwise the results are of doubtful value. Preliminary experiments with *Mytilus byssus* retractor and with slices of the adductor muscles of *Mytilus* and *Anodonta* showed that these tissues were unsuitable as they lost a large part of their potassium during one hour's perfusion with saline. However, the ventricles of both *Anodonta* and *Mytilus* fulfilled the necessary requirements. They are easily isolated (Pilgrim, 1953) and consist of thin sheets of muscle less than 1 mm. thick even when contracted. They maintain an almost constant sodium and potassium content for several hours after isolation (Tables 2, 3), and according to Pilgrim will maintain mechanical activity for several days.

PROCEDURE

The ventricles were isolated by Pilgrim's method and suspended by a fine nylon thread in the active saline which was kept stirred at 15° C. After 2 hr. in the active solution the ventricles were removed, blotted carefully to remove surface fluid and immersed in a current of tracer-free saline in a pyrex tube 5 mm. in diameter. Preliminary experiments showed that the activity associated with the nylon thread was less than 1% of the total activity and was washed away in less than 1 min. The isolated ventricle showed some tendency to roll into a tube so it was essential to maintain a high rate of flow of saline to ensure an adequate washing of all the surfaces of the muscle. The normal rate of flow was 1·0 ml./sec. and tests with coloured solutions showed that the saline was completely replaced about every 15 sec.

Counting was by a G.M. 4 end-window counter and a scaler. Corrections were made for the dead time of the counter, the activity of the background and the decay of the ^{24}Na.

At the end of the experiment the ventricle was analysed for sodium and potassium. The muscle was weighed, dissolved in a drop or two of concentrated nitric acid, evaporated to dryness on a water bath and the residue dissolved in 5 or 10 ml. of distilled water. The sodium and potassium contents were measured by an EEL flame photometer. Experiments were carried out at both 5° and 15° C.

SOLUTIONS

Mytilus blood is similar in composition to sea water but contains more potassium to the extent of about 2 mM/kg. water (Potts, 1954). *Mytilus* ventricle was eluted with filtered sea water to which had been added 2 mM/l. of KCl and 1 mM/l. of

glucose. The final solution contained 480 mM Na/l., 12·1 mM K/l. and 560 mM Cl/l. The pH was in the range 7·8–8·1.

The radioactive saline containing ^{24}Na was prepared by dissolving 20 mg. of irradiated sodium carbonate, pile factor 10, in excess N-HCl and evaporating to dryness and then dissolving in 0·66 ml. of water containing 12·1 mM KCl/l. This produced a solution containing about 570 mM NaCl/l. and 12·1 mM KCl/l. which was then diluted to 10 ml. with the non-radioactive saline to produce a balanced salt solution. The pH of both solutions was always in the range 7·5–8·0.

Anodonta muscle was eluted with a saline containing 14 mM/l. NaCl, 0·5 mM/l. KCl, 5 mM/l. $CaCl_2$, 0·25 mM/l. Na_2HPO_4, and 1 mM/l. glucose. The pH was adjusted, with dilute NaOH, to 7·5. The solution resembles the average composition of *Anodonta* blood except that Cl^- has been substituted for HCO_3^-. *Anodonta* blood normally contains about 10 mM/l. of bicarbonate, but solutions containing so much bicarbonate are unstable and lose CO_2 to the atmosphere.

The radioactive saline was prepared by dissolving 20 mg. of irradiated sodium carbonate, pile factor 10, in 10 ml. of water and adding sufficient 0·1 N-HCl to bring the pH to 7·5. 1 ml. of 12 mM/l. KCl solution and 2 ml. of 65 mM/l. $CaCl_2$ solution were added and the solution was diluted to 26 ml. The final concentrations were: Na, 14·5 mM/l., K, 0·5 mM/l., and Ca, 5 mM/l. The composition closely resembled the average composition of *Anodonta* blood (Potts, 1954) but the solution slowly lost carbon dioxide and became more alkaline. HCl was added at intervals to keep the pH between 7·5 and 8·0.

EXTRACELLULAR SPACE AND INORGANIC COMPOSITION OF THE VENTRICLES

In a previous paper (Potts, 1958) details have been given of the inulin space, water content and inorganic composition of *Mytilus* and *Anodonta* ventricles. The water content of *Mytilus* ventricle is 80·8 ± 0·6 % (w/w) and of *Anodonta* ventricle 87·8 ± 0·8 %. The inulin space of *Mytilus* ventricle is 26·0 ± 3·4 % of the total water content and of *Anodonta* ventricle 30·5 ± 2·5 %. The intracellular concentrations of sodium, potassium and chloride in the two muscles are given in Table 1 together with the average composition of the extracellular fluids. The intracellular chloride content of *Anodonta* ventricle is unfortunately too small to be determined with accuracy.

DIAMETERS OF MUSCLE FIBRES

The average diameters of the muscle fibres of the ventricles of both *Mytilus* and *Anodonta* were determined so that the sodium fluxes through the fibre membranes could be calculated. The ventricles of both *Mytilus* and *Anodonta* are so thin that with good illumination the diameter of the individual fibres can be measured directly in fresh tissue.

The diameters of the muscle fibres were measured as follows. A ventricle was extended under a cover-slip and observed with a $\frac{1}{12}$ in. water-immersion objective.

Table 1. *The sodium, potassium and chloride content of the ventricle and blood of* Mytilus edulis *and of* Anodonta cygnaea (*from Potts*, 1958)

(mM/kg. water content)

	Na	K	Cl
Mytilus			
Whole ventricle	181 ± 10	120 ± 4	190 ± 9
Muscle fibres	73 ± 26	158 ± 4	56 ± 29
Blood	490	12·5 ± 0·2	573
Anodonta			
Whole ventricle	9·5 ± 0·2	10·5 ± 0·6	—
Muscle fibres	7·1 ± 0·7	14·9 ± 0·9	—
Blood	14·7 ± 1·3	0·45 ± 0·014	10·7 ± 1·0

The diameters of twenty adjacent fibres lying in one transect were then measured with a calibrated graduated eye-piece. This was repeated for four ventricles of *Mytilus* and four of *Anodonta.*

Errors may arise for the following reasons. When the fibres are crowded together some confusion may occur between the edges of the fibres. The sites chosen for the transects are necessarily ones where the fibres are well spaced and therefore perhaps not typical of the ventricles. The pressure of the cover-slip may extend the ventricle and therefore slightly reduce the diameters of the fibres. These errors are not likely to be very large and the method is preferable to fixing and staining the tissues which usually involves some shrinkage of the fibres.

The average diameter of the muscle fibres of *Mytilus* ventricle was $9·5 \pm 0·3\,\mu$ and of *Anodonta* ventricle $13·5 \pm 0·7\,\mu$.

THE CONCENTRATIONS OF SODIUM AND POTASSIUM IN THE TISSUES DURING THE COURSE OF THE EXPERIMENTS

The concentrations of sodium and potassium in the ventricles used in the experiments are given in Tables 2 and 3. In some of the experiments both the initial and final sodium and potassium concentrations of the ventricles were measured, but in most experiments the whole of the ventricle was used and so only the final concentrations could be determined. The sodium content of *Anodonta* blood is rather variable and some of the changes in the sodium content of the ventricle during the course of the experiment may be caused by differences between the sodium content of the blood and the eluting saline. The results in Tables 2 and 3 show clearly that the ionic contents of the ventricles at the end of the experiments were similar to those of fresh material. In particular there is practically no fall in the potassium content of either *Mytilus* or *Anodonta* ventricles. The sodium content of the eluting saline for *Mytilus* ventricle, 480 mM/l., was not identical with the sodium content of the blood of *Mytilus* quoted in Table 1, namely 490 mM/kg. water. Any effect this may have had on the intracellular concentration of sodium has been neglected in the subsequent calculations in which it has been assumed that the intracellular concentrations of the ions are those given in Table 1.

Table 2. *The sodium and potassium content of the ventricles of* Mytilus edulis *used in the experiments*

(mM/kg. water)

Mytilus (no.)	Before experiment		After experiment	
	Na	K	Na	K
1	194	128	230	145
2	261	119	193	155
3	132	122	199	125
4	—	—	279	105
5	—	—	185	150
6	—	—	268	125
7	—	—	203	133
8	—	—	192	137
Mean	195	123	219	134

Table 3. *The sodium and potassium content of the ventricles of* Anodonta cygnaea *used in the experiments*

(mM/kg. water)

Anodonta (no.)	Before experiment		After experiment	
	Na	K	Na	K
1	—	—	11·8	14·4
2	10·8	7·6	9·8	8·8
3	7·6	10·4	10·8	5·5
4	10·2	10·5	9·0	12·0
5	8·6	10·9	12·4	11·1
6	11·7	8·9	7·8	8·9
7	8·2	10·3	9·0	8·6
8	8·3	10·1	7·4	10·4
9	—	—	7·0	9·2
10	—	—	10·6	6·8
Mean	9·3	9·8	9·6	9·6

SODIUM FLUXES IN *MYTILUS* AND *ANODONTA* VENTRICLES

At 5° C. in all experiments the time course of the decay of the activity of the muscle, when eluted with a non-radioactive saline, approximates to the sum of two exponentials which may be represented by the expression $Ae^{-K_1t}+Be^{-K_2t}$. This is clearly seen when the results are plotted semi-logarithmically (Figs. 1, 3). The more rapidly declining part of the activity may be attributed to the sodium in the extracellular spaces, while the more slowly exchanging part may be attributed to the intracellular sodium (see Appendix). At 5° C. the distinction between the two parts is quite clear and after about 10 min. the activity in the extracellular sodium has become insignificant and the activity of the muscle declines as a simple exponential function of the time, Be^{-K_2t}. At 15° C. (Fig. 2) the rate of exchange of sodium between the fibres and the extracellular fluid is considerably faster than

at 5° C. (Fig. 1), but the rate of diffusion from the extracellular spaces is not appreciably altered and the distinction between the two phases is not so clear. The rate of turnover of sodium inside the fibres of *Anodonta* muscle is rather faster than

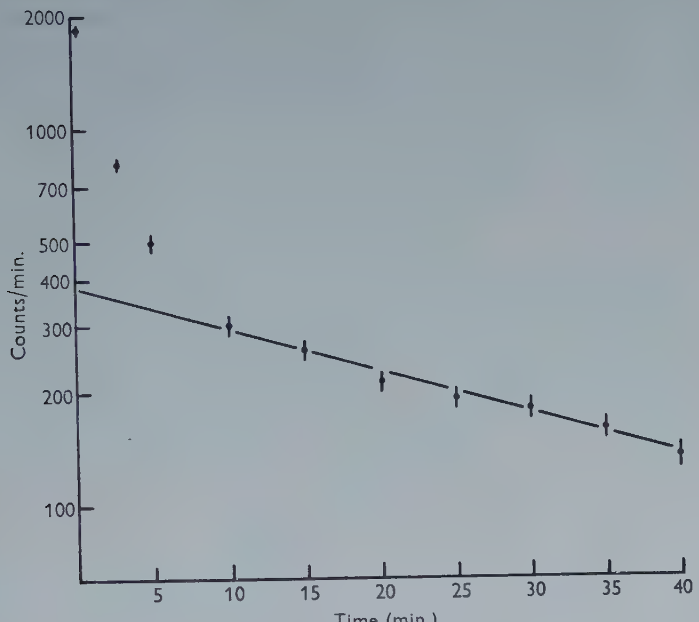

Fig. 1. The loss of ^{24}Na from *Mytilus* ventricle no. 2 when washed in inactive saline at 5° C. The straight line represents the loss from the fibres.

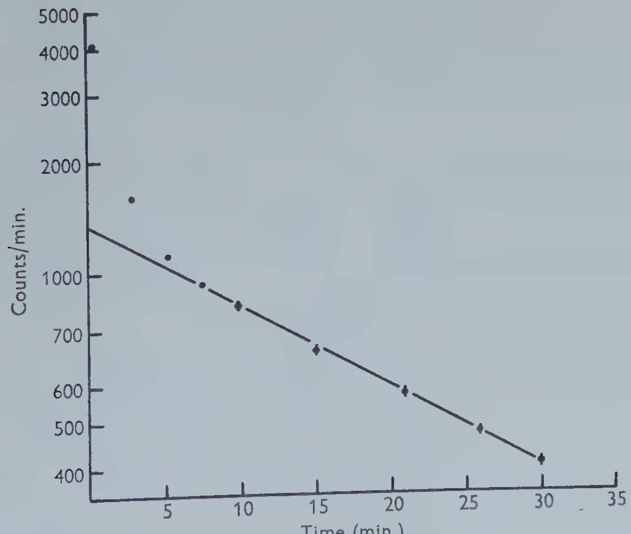

Fig. 2. The loss of ^{24}Na from *Mytilus* ventricle no. 10 when washed in inactive saline at 15° C. The straight line represents the loss from the fibres.

in *Mytilus* muscle and at 15° C. the extracellular and intracellular parts are not distinguishable, although at 5° C. they are still clear (Fig. 3). For this reason the sodium flux in *Anodonta* muscle at 15° C. could not be measured.

After the first ten minutes, in all experiments, the activity declines exponentially and so all the points lie on a straight line, Be^{-K_2t}. By extrapolating this line back to $t = 0$, B can be obtained. The difference between the experimental curve and the straight line represents, to a first approximation, the diffusion of sodium from the extracellular fluid. The diffusion of a substance into, or out of, a thin sheet has

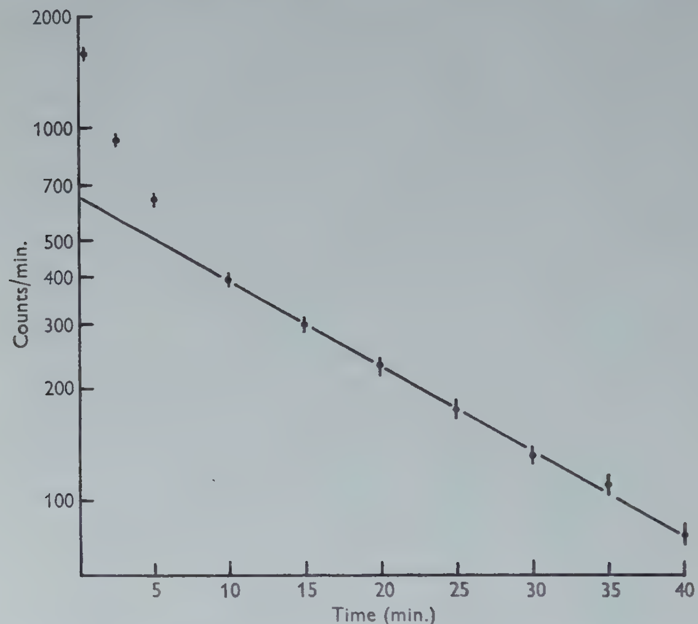

Fig. 3. The loss of ^{24}Na from *Anodonta* ventricle no. 4 when washed with inactive saline at 5° C. The straight line represents the loss from the fibres.

been investigated by Hill (1928). The rate of decline is most rapid at first but the fall becomes a simple exponential after about one-quarter of the substance has diffused out. In these experiments A and K_1 have been calculated on the assumption that the loss from the extracellular spaces is a simple exponential function of time since the sodium in the extracellular water declined so rapidly that the exact shape of the curve could not be determined.

The results of the experiments are summarized in terms of K_1, K_2, A and B in Table 4. In all cases the rate constant K_1 is several times larger than K_2. K_1 is almost independent of temperature while K_2 is lower at 5° C. than at 15° C.

CALCULATION OF SODIUM FLUXES IN MUSCLE FIBRES

The analysis of the efflux of sodium from a muscle containing both an extracellular and an intracellular phase is complicated because most of the fibres communicate not with the tracer-free saline but with the intracellular fluid which contains a

degree of radioactivity depending on K_1, K_2, A and t. Some of the ions leaving the fibres re-enter the fibres before they are swept away.

For this reason the apparent rate constant for the efflux of sodium from the fibres, K_2, will be less than the real rate constant k_2.

The mathematics of this system has been discussed by Harris & Burn (1949) and Keynes (1954) and Keynes has derived equations relating k_2 to K_2. Unfortunately these equations involve the thickness of the muscle and both *Mytilus* and *Anodonta* ventricles are so variable in thickness that it is convenient to rearrange the equations to eliminate the thickness of the muscle and some other quantities (see Appendix). k_2 may then be calculated from K_1, K_2, C_0, C_i and ϵ, where C_0 and C_i are the extracellular and intracellular concentrations of sodium and ϵ is the fraction by volume of the extracellular fluid.

The equations are only strictly applicable to a plane sheet of muscle and are not completely accurate even for that simple case, but the corrected values k_2 are probably to be preferred to the uncorrected K_2. The corrected values of the rate constant of the efflux from the intracellular fraction, k_2, are given in Table 4.

Using the corrected values of k_2, M, the flux of sodium through unit area of fibre surface can be calculated from the expression

$$M = k_2 \frac{V}{A} C_i \quad \text{(Keynes, 1954, eq. 1).}$$

Table 4. *Constants of the efflux of sodium from lamellibranch ventricles. For details see text*

No.	Temp.	A cts/min.	B cts/min.	$\frac{B}{A+B}$	K_1 hr.$^{-1}$	K_2 hr.$^{-1}$	k_2 hr.$^{-1}$
Mytilus	5° C.						
2		1476	380	0·204	11·1	1·50	1·69
3		1790	560	0·207	8·0	2·61	3·08
8		348	520	0·60	13·9	2·52	2·74
4		1830	1260	0·41	6·9	2·37	2·80
9		1260	930	0·42	5·3	1·64	1·91
Mean							2·44
5	15° C.	1780	1100	0·38	5·9	4·87	7·0
6		566	302	0·35	5·8	4·53	6·7
7		589	151	0·20	6·4	3·88	5·2
10		2620	1355	0·34	10·0	3·03	3·4
Mean				0·35			5·6
Anodonta	5° C.						
1		890	1510	0·63	8·3	3·80	6·9
2		1780	1160	0·39	6·9	2·66	4·4
4		930	650	0·41	6·4	3·08	5·5
6		510	1670	0·77	8·1	3·18	5·3
7		623	722	0·54	6·9	3·42	6·5
8		420	580	0·58	6·6	3·33	6·5
9		306	258	0·46	5·2	1·51	2·2
10		2250	3850	0·63	8·3	1·92	2·6
11		2900	3050	0·51	12·9	1·51	1·7
Mean				0·55			4·6

For an infinite cylinder $V/A = \frac{1}{2}r$, where r is the radius of the cylinder.

For *Mytilus* $r = 4{\cdot}75\,\mu$,

$C_i = 73$ mM/kg. water,

$k_2 = 2{\cdot}44$ hr.$^{-1}$ at $5°$ C.

$= 5{\cdot}6$ hr.$^{-1}$ at $15°$ C.

Hence $M = 12 \times 10^{-6}$ mM cm.$^{-2}$ sec.$^{-1}$ at $5°$ C.

$= 27 \times 10^{-6}$ mM cm.$^{-2}$ sec.$^{-1}$ at $15°$ C.

For *Anodonta*, $r = 6{\cdot}75\,\mu$,

$C_i = 7{\cdot}2$ mM/kg. water,

$k_2 = 4{\cdot}6$ hr.$^{-1}$ at $5°$ C.

Hence $M = 3{\cdot}1$ mM-cm.$^{-2}$ sec.$^{-1}$ at $5°$ C.

THE ENERGY REQUIRED FOR SODIUM EXTRUSION

The sodium removed from the muscle is secreted against both a concentration gradient, E_{Na}, and an electrical potential, E_v.

If it is assumed that the efflux of sodium is entirely an active extrusion, uncomplicated by an exchange diffusion, and if it is also assumed that the activity coefficient of the sodium inside the fibres is the same as the activity coefficient of the sodium outside the fibres, then the secretory work, W, is given by the expression

$$W = k_2 F(E_{\mathrm{Na}} + E_v), \tag{1}$$

where

$$E_{\mathrm{Na}} = \frac{RT}{F} \ln \left[\frac{\mathrm{Na}_0}{\mathrm{Na}_i}\right], \tag{2}$$

R is the universal gas constant, F the faraday, Na_i the intracellular concentration of sodium and Na_0 the extracellular sodium concentration.

E_v has not been measured in lamellibranch muscle but can be calculated approximately from the potassium and chloride concentration gradients across the sarcolemma. The distribution of potassium and chloride in *Mytilus* ventricle is close to a Donnan equilibrium in which $\mathrm{K}_i/\mathrm{K}_0 = \mathrm{Cl}_0/\mathrm{Cl}_i$ (Potts, 1958), and the resting potential may be calculated from the equation

$$E_v = \frac{RT}{F} \ln \left[\frac{\mathrm{K}_i}{\mathrm{K}_0}\right]. \tag{3}$$

Unfortunately it is not possible to measure accurately the intracellular concentration of chloride in *Anodonta* ventricle and there is some evidence (Potts, 1958) that the potassium-ion concentration gradient between the inside and the outside of some lamellibranch muscle fibres is greater than the concentration gradient of chloride ions. In these cases the electrical potential of the fibres may be less than the equilibrium potential of the potassium. However, in the absence of further information, it will be assumed that the potassium is in equilibrium with the resting potential.

After substituting in equation (1) for E_{Na} and E_v with the aid of equations (2) and (3)

$$W = k_2\mathrm{Na}_i\, RT\left(\ln\left[\frac{\mathrm{Na}_0}{\mathrm{Na}_i}\right]+\ln\left[\frac{\mathrm{K}_i}{\mathrm{K}_0}\right]\right).$$

For *Mytilus*,

$$\begin{aligned}
\mathrm{Na}_i &= 73 \text{ mM/kg. water,}\\
\mathrm{Na}_0 &= 480 \text{ mM/kg. water,}\\
\mathrm{K}_i &= 150 \text{ mM/kg. water,}\\
\mathrm{K}_0 &= 12{\cdot}1 \text{ mM/kg. water,}\\
k_2 &= 2{\cdot}44 \text{ hr.}^{-1} \text{ at } 5^\circ \text{ C.}\\
k_2 &= 5{\cdot}6 \text{ hr.}^{-1} \text{ at } 15^\circ \text{ C.}
\end{aligned}$$

Hence

$$\begin{aligned}
W &= 440 \text{ cal./kg. fibre water/hr. at } 5^\circ \text{ C.}\\
&= 1030 \text{ cal./kg. fibre water/hr. at } 15^\circ \text{ C.}
\end{aligned}$$

1 kg. of *Mytilus* ventricle contains 807 g. of water of which 26% is extracellular, so that 1 kg. of ventricle contains only 600 g. of fibre water. Hence

$$\begin{aligned}
W &= 0{\cdot}265 \text{ cal./g./hr. at } 5^\circ \text{ C.}\\
&= 0{\cdot}62 \text{ cal./g./hr. at } 15^\circ \text{ C.}
\end{aligned}$$

For *Anodonta*,

$$\begin{aligned}
\mathrm{Na}_i &= 7{\cdot}2 \text{ mM/kg. water,}\\
\mathrm{Na}_0 &= 14{\cdot}0 \text{ mM/kg. water,}\\
\mathrm{K}_i &= 14{\cdot}9 \text{ mM/kg. water,}\\
\mathrm{K}_0 &= 0{\cdot}5 \text{ mM/kg. water,}\\
k_2 &= 4{\cdot}6 \text{ hr.}^{-1}.\\
\text{Water content} &= 878 \text{ g./kg. muscle.}\\
\text{Extracellular space} &= 30{\cdot}5\,\% \text{ water content.}
\end{aligned}$$

Hence

$$W = 0{\cdot}046 \text{ cal./g./hr.}$$

DISCUSSION

Most of the previous measurements of sodium fluxes have been made either on vertebrate tissues, in which the extracellular concentration of sodium is of the order of from 100 to 150 mM/l. or on *Sepia* axons in which surface/volume ratio of the cells is much smaller than in the lamellibranch muscle fibre, so those results are not exactly comparable with the results reported here. The sodium fluxes per unit area of the fibre surface of the lamellibranch muscles are of the same order as those reported for vertebrate muscles. At 5° C. the sodium flux through *Mytilus* ventricle fibre is about 12×10^{-6} mM/cm.^{2}/sec. and at 15° C. is about 27×10^{-6} mM/cm.^{2}/sec. Harris & Burn (1949) and Keynes (1954) reported sodium fluxes of 10 and $5{\cdot}4 \times 10^{-6}$ mM/cm.^{2}/sec. at 16° and 17° C. respectively, through the fibres of the frog sartorius. The difference between the frog and the marine lamellibranch may well arise from the much greater concentration of sodium in *Mytilus* blood. In *Anodonta* the sodium flux, $3{\cdot}1 \times 10^{-6}$ mM/cm.^{2}/sec. is less than in the frog. In the frog sartorius the rate constant k_2 for the exchange of sodium is much smaller

than in *Mytilus* but the fibres are much larger with a diameter of about 80μ. In the rat diaphragm the fibres are only about 20μ in diameter and k_2 is 3·75 hr.$^{-1}$ at 37° C. (Creese, 1954). In the rat diaphragm the sodium flux/unit area of fibre surface at 37° C. is as high as in *Mytilus* at 15° C., 27 mM/cm.^{2}/sec. The 20° C. temperature difference compensates for the fourfold difference in sodium concentrations in the external fluids. The only measurements of sodium fluxes in tissues for marine animals are of the giant axons of *Sepia* where the sodium flux through the surface of the axon is even larger than in *Mytilus* muscle and amounts to 40 mM/cm.^{2}/sec. during recovery from stimulation (Hodgkin & Keynes, 1954).

Although the sodium fluxes per unit surface area of lamellibranch muscles are comparable with those reported for other tissues, the theoretical energy required to maintain the flux is much larger in *Mytilus* than any previously reported. This is the result of the combination of a very narrow fibre, and hence a large surface volume ratio with a high concentration of sodium in the blood. The theoretical energy requirements of *Mytilus* muscle at 15° C. is 0·62 cal./g./hr. Keynes & Maisel (1954) calculated that in frog muscle only about 0·04 cal./g./hr. were required. Hodgkin & Keynes (1954) estimated that *Sepia* axons required about 0·08 cal./g./hr. In *Anodonta* muscle, where the ambient sodium concentration is much lower, only about 0·046 cal./g./hr. are required.

Levi & Ussing (1948) considered that the efflux of sodium from the frog sartorius was too large to represent an active process and suggested that part of it might be caused by an exchange diffusion requiring no energy. Hodgkin & Keynes (1955) have shown that in *Sepia* axons the sodium flux was not reduced even in the absence of external sodium under which conditions exchange diffusion would not occur. However, more recently Swan & Keynes (1956) have shown that the substitution of choline for sodium reduced the sodium efflux from frog muscle by more than half. In this case the energy requirement would be correspondingly reduced.

It is probable that in the lamellibranchs exchange diffusion is responsible for part of the efflux, but the apparent energy requirements of *Mytilus* ventricle are more than five times as great as those of *Anodonta* ventricle and exchange diffusion, if it occurs, is likely to take place in *Anodonta* ventricle as well. It is, therefore, very probable that the energy required for sodium extrusion is substantially greater in the marine species.

Measurements of the oxygen consumption of the two muscles, which will be reported in a later paper, show that in both animals the metabolic energy available is about twice as great as the apparent energy requirements for sodium extrusion, but the metabolic rate of the marine animal is several times greater than that of the freshwater animal.

This has a number of interesting implications in the field of osmotic regulation. It suggests, for example, that a freshwater animal may perform less ionic work than a marine animal; for although it has to perform a certain amount of ionic work at the body surface it may be saved a large amount of ionic work at the surface of each cell. Conversely the many marine animals which maintain a salt concentration

in the blood which is less than that of sea water, for example teleosts, selachians, lampreys, sturgeons, grapsoid crabs and many shrimps, may be more efficient than otherwise appears.

APPENDIX

DERIVATION OF k_2 FROM K_2

Keynes (1954) derived the following equations:

If U is the rate of loss of activity if all the fibres are exposed to non-radioactive saline and U' is the observed rate of loss in a plane sheet of muscle, then

$$\frac{U'}{U} = \frac{\lambda}{b}\tanh\frac{b}{\lambda} \quad \text{(Keynes, eq. 17)}$$

where b is half the thickness of the muscle and λ is the factor by which the distance any particle has to travel from the surface to any point inside is increased by obstacles, the muscle fibres. Also

$$\lambda^2 = \frac{\epsilon}{1-\epsilon}\frac{V}{A}\frac{C_0}{M}D' \quad \text{(Keynes, eq. 9)},$$

where ϵ is the fraction, by volume, occupied by the extracellular fluid, V/A is the volume/area ratio of the muscle fibres, C_0 is the molar concentration of extracellular sodium, M is the flux of sodium through the muscle fibre surface in mole/cm.^{2}/unit time and D' is the quantity of sodium diffusing through area $1/\epsilon$ of the muscle in unit time under unit concentration gradient.

λ and D' can be eliminated as follows.

If $t_{0\cdot5}$ is the half time of washing out of radioactivity from the extracellular phase

$$t_{0\cdot5} = \frac{0\cdot28b^2}{D'} \quad \text{(Keynes, eq. 5)},$$

but

$$t_{0\cdot5} = \frac{\ln 2}{K_1} = \frac{0\cdot693}{K_1},$$

therefore

$$D' = \frac{0\cdot28b^2K_1}{0\cdot693} = 0\cdot404b^2K_1.$$

Substituting for D' in Keynes, eq. 9

$$\frac{\lambda^2}{b^2} = 0\cdot404\frac{\epsilon}{1-\epsilon}\frac{V}{A}\frac{C_0}{M}K_1.$$

But

$$M = k_2\frac{V}{A}C_i \quad \text{(Keynes, eq. 1)},$$

where C_i is the concentration of sodium inside the fibres and k_2 is the rate constant of exchange of sodium between the fibres and the extracellular fluid. Hence

$$\frac{\lambda^2}{b^2} = 0\cdot404\frac{\epsilon}{1-\epsilon}\frac{C_0}{C_i}\frac{K_1}{k_2} \tag{X}$$

and

$$\frac{U'}{U} = \frac{K_2}{k_2} = \frac{\lambda}{b}\tanh\frac{b}{\lambda}. \tag{Y}$$

ϵ, C_0, C_i, K_1 and K_2 are known, hence by successive approximations k_2 can be determined.

In equation (X) above,

$$\frac{\epsilon}{1-\epsilon}\frac{C_0}{C_i} = \frac{\text{sodium in extracellular phase}}{\text{sodium in intracellular phase}} \simeq \frac{A}{B}.$$

In Table 4, k_2 has been calculated from values of ϵ, C_0 and C_i derived by chemical analysis. From the figures published by Potts (1958) $\epsilon C_0/(1-\epsilon)C_i = 1{\cdot}79$ for *Mytilus* and 0·75 for *Anodonta*. Only slightly different values of k_2 are obtained if A/B is used instead. Hence k_2 can be determined entirely from the experimental data.

The intracellular fraction of sodium in *Mytilus* ventricle is 30 %, calculated from the chemical data. When calculated as $B/(A+B)$ it is slightly higher, 35 %. For *Anodonta* the corresponding values are 52 and 55 %. The similarity of these values confirms the identity of the faster moving fraction with the extracellular fraction and the slower with the intracellular fraction.

SUMMARY

1. Measurements have been made, using ^{24}Na, of the efflux of sodium from the isolated ventricles of *Mytilus edulis* and *Anodonta cygnaea*.

2. In order to determine the efflux of sodium from the muscle fibres it is necessary to correct for the efflux of sodium from the extracellular space. It was not practicable to make such a correction to the results on *Anodonta* at 15° C.

3. The mean rate constants and effluxes of sodium from the muscle fibres are

Mytilus at 5° C., 2·44 hr.$^{-1}$; 12×10^{-6} mM/cm.^{2}/sec.
at 15° C., 5·6 hr.$^{-1}$; 27×10^{-6} mM/cm.^{2}/sec.

Anodonta at 5° C., 4·6 hr.$^{-1}$; $3{\cdot}1 \times 10^{-6}$ mM/cm.^{2}/sec.

4. The energy required for sodium extrusion, assuming it is entirely an active process, is:

Mytilus at 5° C., 0·26 cal./g./hr.
at 15° C., 0·62 cal./g./hr.

Anodonta at 5° C., 0·046 cal./g./hr.

I am indebted to the Director of the Marine Laboratory, Plymouth, for facilities given to me during my visits. I am also grateful to Dr B. C. Abbott of the Plymouth Laboratory for help and advice and to Dr J. C. Bevington of the Chemistry Department, Birmingham University, for the loan of a counter and scaler.

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THE RESPIRATION OF FRESHWATER SNAILS

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INTRODUCTION

The chief aim of this study was to investigate the oxygen consumption of some Danish freshwater snails under varying oxygen concentrations in order to elucidate, if possible, the capacity of the species to exist in nature under unfavourable respiratory conditions.

The first step was to investigate the relation between respiration and starvation; knowledge of this is a prerequisite for an evaluation of oxygen consumption at various times after collection of the animals.

Furthermore, the respiration of the species at varying temperatures has been investigated in order to make possible a comparison between results obtained at different temperatures of the water.

The oxygen consumption of the snails varies with the weight of the individuals. In the case of the limpet *Ancylus fluviatilis* it was found earlier that the oxygen consumption is proportional to about $w^{0.73}$ where w is the live weight (Berg, Lumbye & Ockelmann, 1958). The relation of oxygen consumption to weight for the individuals of each species was also investigated.

After the preliminary studies on the relation of respiration to starvation, varying temperature and weight of individuals, the oxygen consumption in relation to oxygen content of the water was studied.

All experiments were carried out in 1957. The animals were not narcotized and they were all collected from their localities just before the experiments. The observed rate of respiration must, therefore, be regarded as about the same as that in nature under similar conditions. This is not a basal or standard respiration, but an *active* respiration. The snails move only slowly in the respiratory bottles, but still it is an active oxygen consumption whose relation to environmental conditions has been studied.

A few words, which characterize the localities from where the experimental animals were collected, will be appropriate.

Physa fontinalis (L.), *Myxas glutinosa* (O. F. Müller) and *Lymnaea auricularia* (L.). From the sandy shore of the clear, slightly eutrophic lake, Slåensø (Jutland), at a depth of 0·2–1 m.; the first mentioned in April, the others in May.

Lymnaea pereger (O. F. Müller) and *L. palustris* (O. F. Müller). From stones on the shore of the eutrophic lake, Esrom Sø (Sealand) at a depth of 0–0·2 m.; June. Also in August among algae washed ashore.

Bithynia tentaculata (L.). From a eutrophic, slightly humic pond in a beech wood, mostly on branches and in the vegetation, to a depth of 1 m.; July, August and October.

Valvata piscinalis (O. F. Müller). In the littoral zone of Esrom Sø, at a depth of 0·5–2 m. on sandy and gravelly bottom; September and October.

Bithynia leachi (Sheppard). From Esrom Sø, at a depth of 1–2 m., partly on gravelly bottom at the shore and partly on mud smelling of H_2S in a yachting harbour, October and November.

Concerning the localities of species studied earlier the following data are given:

Acroloxus lacustris (L.). From two eutrophic, slightly humic localities, Karlsø and Torkerisø (Sealand), on stems of plants and in cavities below withered leaves. May–June and September–October (Berg, 1952).

Theodoxus fluviatilis (L.). From fresh water: On stones at a depth of *c.* 0·5 m. in the eutrophic lake Borresø (Jutland); June. From brackish water: On stones at a depth of 0·3–0·5 m. in Ringkøbing inlet (Jutland), salinity *c.* 9–11‰; July (Lumbye, 1958).

Potamopyrgus jenkinsi (Smith). From fresh water: In a marlpit at Kolstrup (Jutland) with a freshwater fauna without any brackish-water elements; July. From brackish water: The same locality Ringkøbing inlet, as mentioned above (Lumbye, 1958).

METHODS

The oxygen determinations were made by a polarometric method developed by Bartels and used earlier for similar purposes (cf. Berg, 1953; Berg *et al.* 1958; Lumbye, 1958).

Where not otherwise indicated the experiments were carried out as follows. The animals were collected in nature, put in a Dewar vessel with water from the locality and brought to the laboratory. The journey lasted *c.* $\frac{1}{2}$ hr. The animals were then placed in aerated water in a thermostatic bath, and the experiments started almost at once.

The experiments were made in closed respiration chambers, that is, in bottles containing 4–12 ml. of aerated water, the volumes of which were known exactly. The oxygen content of the water was measured before the experiment. During the experiment the bottles were placed in darkness in the water bath. The duration of an experiment was usually 1 hr. After the experiment the oxygen content of the water was measured again and this was usually 60–70% of the oxygen content at the beginning. The difference between the two oxygen determinations is the oxygen consumed.

The snails were dried on filter paper, weighed and killed in boiling water; their shells were then dried and weighed. The difference between the two weights is the live weight of the animals.

The oxygen consumption found in the experiments was calculated in μl. per hr. per individual of a particular live weight, the *standard individuals.* This is an individual of about the mean of the weight variation of the particular species. This seems more correct than calculating the oxygen consumption per gram, as such a figure may vary according to the size of the animal, large ones often having a lower oxygen consumption per gram than smaller ones.

Every experiment on the oxygen consumption of a standard individual was based upon a series of 5–6 separate determinations of the type described above (cf. fig. 1,

p. 46, Berg *et al.* 1958; and Berg, 1953; Mann, 1956; Lumbye, 1958). The animals selected for experiments might vary from a few to several hundred milligrams, but all individuals placed in the same respiration bottle were carefully selected so as to be of the same size. Several small, or some few medium-sized, or one or two large snails were put in every respiration bottle. Thus often twenty to thirty specimens were used in every experiment.

All the pulmonates which were studied had their lungs filled with water.

Accuracy of measurements. On the basis of several series of experiments of the same type as previously described for the limpet *Ancylus fluviatilis* the accuracy of the measurements was computed (Berg *et al.* 1958, pp. 47–49). It was found that the standard deviation for the respiration of a standard individual is $\pm 2\%$. Thus there is a 95 % probability that a new experiment of the same type would give a result which would at most deviate *c.* $\pm 4\%$ from the average of a series of experiments. A somewhat similar accuracy must be expected of the measurements in this paper. But because it was also found in the experiments with *A. fluviatilis* that the cause of the variation of the single determinations is essentially of a physiological nature, not so much an experimental error, it may be supposed that in experiments under the same conditions with other snails the results may vary somewhat more than $\pm 4\%$ from the average.

OXYGEN CONSUMPTION IN RELATION TO STARVATION

The purpose of these experiments was to see if the oxygen consumption decreased after collection of the animals in nature, owing to partial or total starvation. If so, it might be necessary to pay attention to this in the evaluation of the results of other experiments carried out over a period of several hours after collection. In the case of *A. fluviatilis* it was found that starvation markedly influenced respiration (Berg *et al.* 1958).

The animals were collected and carried to the laboratory in vacuum flasks. They were transferred to respiration bottles, usually in the water from which they were collected, and kept in these bottles in a thermostatic bath at the same temperature ($\pm$ 1° C.) as that of the locality from which they had been collected. The oxygen consumption was determined at intervals over 8 or more hours and between measurements the water was aerated.

The results are shown in Fig. 1, where the oxygen consumption per live weight of the standard individuals is indicated along the ordinate. The abscissa shows the time elapsed after collection of the animals. According to the different sizes of the species, the live weight of the standard individuals varies from species to species.

Fig. 1 shows that *Lymnaea pereger*, *Myxas glutinosa*, *Bithynia tentaculata*, *Valvata piscinalis* and possibly *Physa fontinalis* and *Lymnaea auricularia* (only two determinations) show only a very little or no decrease of oxygen consumption in the period investigated. But in *Lymnaea palustris* and *Bithynia leachi* a distinct decrease has been found, and this may be taken into account when the relation of respiration to temperature is discussed.

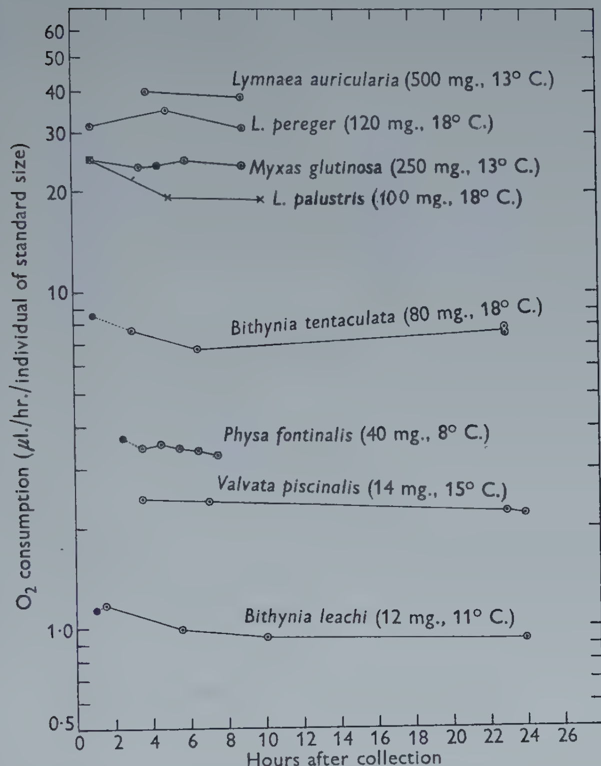

Fig. 1. Oxygen consumption of freshwater gastropods in relation to partial or complete starvation. The standard live weight of the species and the experimental temperatures are indicated after the names of the snails. The filled circles show results from supplementary experiments.

THE OXYGEN CONSUMPTION IN RELATION TO TEMPERATURE

It was found earlier that the oxygen consumption of *Ancylus fluviatilis* during a gradual increase of temperature from 11° to 18° C. followed Krogh's curve in the main, but not always (Berg *et al.* 1958).

The relation of the oxygen consumption to the temperature was studied during the present experiments, the temperature being increased *c.* 1° per hr. from the temperature at which the animals were collected in nature. In a few cases the oxygen consumption was also investigated during a gradual decrease of the temperature. When a particular change of temperature had been completed, a respiration experiment was carried out at a constant temperature and in the usual way.

The results are shown in Fig. 2 and for comparison a Krogh's curve drawn on a logarithmic scale is also shown. Besides the observed values of *Bithynia leachi*, values are given computed after correction for starvation in accordance with the results from Fig. 1. For the other species no correction has been made. (No experiment of this type was carried out with *Lymnaea palustris*.)

According to Krogh's curve about a 100% increase of oxygen consumption

follows an increase of the temperature from 11° to 18° C. For this temperature increase the species investigated show, according to Fig. 2, the following increases of oxygen consumption:

	%
Myxas glutinosa	*c.* 90
Physa fontinalis	*c.* 90
Bithynia leachi (corrected)	*c.* 85
B. tentaculata	*c.* 70
Lymnaea pereger	*c.* 65

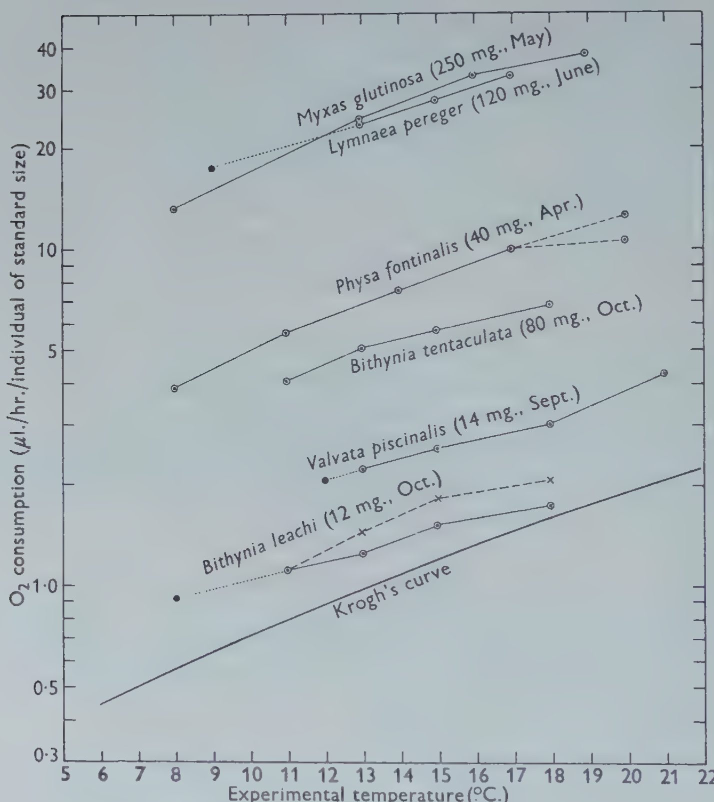

Fig. 2. Oxygen consumption of freshwater gastropods in relation to a gradual increase of the experimental temperature from the temperature of the localities in the field. As a supplement are also added some results of quite similar experiments (filled circles). In *Bithynia leachi* the full-drawn curve indicates the values found in the experiments, the broken curve and crosses the values corrected for starvation according to the experiments shown in Fig. 1.

When the temperature changes from 13° to 21° C. *Valvata piscinalis* shows an increase of 80 % of that shown by the corresponding part of Krogh's curve.

Thus during gradual increase of temperature the snails increased their oxygen consumption by *c.* 65–90 % of the increase according to Krogh's curve. In the case

of *Myxas glutinosa* and *Physa fontinalis* the increase of rate of respiration must be regarded as nearly the same as that found by Krogh and applying to other animals, because the uncertainty of the two curves must be taken into consideration.

Deviations from Krogh's curve may be caused by a change of the state of activity in the experimental animals, and here it should be remembered that oxygen consumption was not determined under conditions of basal metabolism.

These results make it possible to compare the oxygen consumptions found at various temperatures, but the differences found between the snail species can hardly at the present moment be connected with particular ecological conditions.

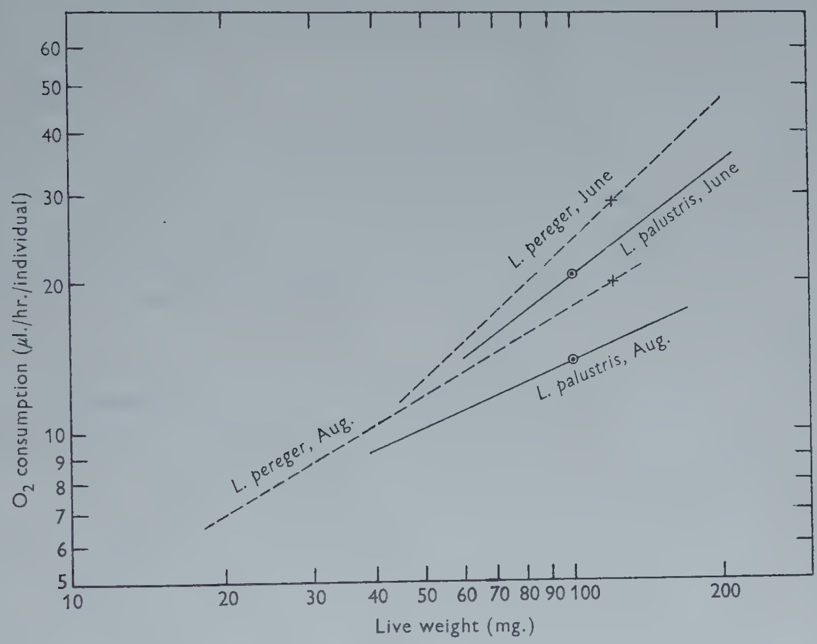

Fig. 3. Intraspecific variation of oxygen consumption in relation to size of individuals (live weight) of *Lymnaea palustris* (full-drawn lines) and *L. pereger* (broken lines). The experiments were carried out in June and August as indicated on the regression lines. The projection of the regression lines on the abscissa shows the variation in the size of the experimental animals.

OXYGEN CONSUMPTION IN RELATION TO SIZE (LIVE WEIGHT)

(*a*) *Intraspecific variation*

The experiments on the relation of oxygen consumption to the weight of the snails showed that in the same species this relation may differ at various seasons. Examples of such intraspecific variation are shown in Fig. 4. In this figure the regression lines are, however, drawn on a *graphical estimate*, as described above. This gives satisfactory determinations of the oxygen consumption of the standard individuals. But in order to show that there exists a significant variation in the relation of oxygen

consumption to live weight two series of experiments with *Lymnaea palustris* and *L. pereger* respectively were computed statistically and compared (Fig. 3).

Lymnaea palustris. The first series of experiments was carried out in June and included three experiments of the usual type, each of which was made up of five determinations of the oxygen consumption of individuals of varying sizes. The equation of the common regression line of these experiments is computed to be $\log y = -0{\cdot}2065 + 0{\cdot}7609 \log x$, and the slope of the regression line, $b = 0{\cdot}761$, has the standard deviation $s = \pm 0{\cdot}088$.

The second series of experiments with *L. palustris* was carried out in August and included four experiments of the usual type, each of which comprised six determinations. The equation of the common regression line of these experimental results is $\log y = +0{\cdot}2421 + 0{\cdot}4516 \log x$, and the slope of the line, $b = 0{\cdot}452$, has the standard deviation $s = \pm 0{\cdot}070$.

The two regression lines are shown in Fig. 3 (unbroken line). The difference between their slopes, $0{\cdot}761 - 0{\cdot}452 = 0{\cdot}309$, is significant ($97{\cdot}5\% < P < 99{\cdot}0\%$, where P is the probability), as shown by the above-mentioned standard deviations.

L. pereger. The first series of experiments with this species was carried out in June and included four experiments, each of which was made up of six determinations. The equation of the common regression line of these experiments is found to be $\log y = -0{\cdot}4905 + 0{\cdot}9379 \log x$, and the slope $b = 0{\cdot}938$ has the standard deviation $s = \pm 0{\cdot}036$.

The second series of experiments was carried out in August and included five experiments, each of which comprised six determinations. In this case the equation of the common regression line is $\log y = 0{\cdot}0807 + 0{\cdot}5856 \log x$, and the slope $b = 0{\cdot}586$ has the standard deviation $s = \pm 0{\cdot}105$.

The last-mentioned two regression lines are also drawn in Fig. 3 (broken). The difference between their slopes, $0{\cdot}352$, is highly significant ($P > 99{\cdot}95\%$), as shown by their standard deviations.

All in all the experiments with *L. palustris* and *L. pereger* have shown that the dependence of the oxygen consumption on the size (live weight) of the individuals varies seasonally. Thus the relation, oxygen consumption to body size, is not a fixed, unchangeable quantity characteristic of all species as supposed by Bertalanffy (1957). He thinks that there are three metabolic types. In the first type the metabolic rate is proportional to the surface or the 2/3 power of the weight, in the second type the rate is proportional to the weight itself, and in his third type the metabolic rates are intermediate between proportionality to weight and proportionality to surface area. Bertalanffy thinks that only one of the three possible relations mentioned is characteristic of any one species. After a survey of the available observations he draws the conclusion (*loc. cit.* p. 220) that in general it can be said that the 'metabolic type', i.e. the relation of metabolic rate to body size, is a physiological characteristic of the species or group concerned.

Furthermore Bertalanffy finds that as there are different metabolic types, so also there are different growth types, which are distinguished by the mode of growth as expressed in growth curves. These show the growth rate of the various species, and

Bertalanffy thinks the growth types may be correlated with his three metabolic types. If this were true, then the observed seasonal variation in metabolic type would imply a seasonal variation in the type of growth rate.

The seasonal variation of the relation between oxygen consumption and body size cannot be caused by the inclusion of both juvenile and sexually mature

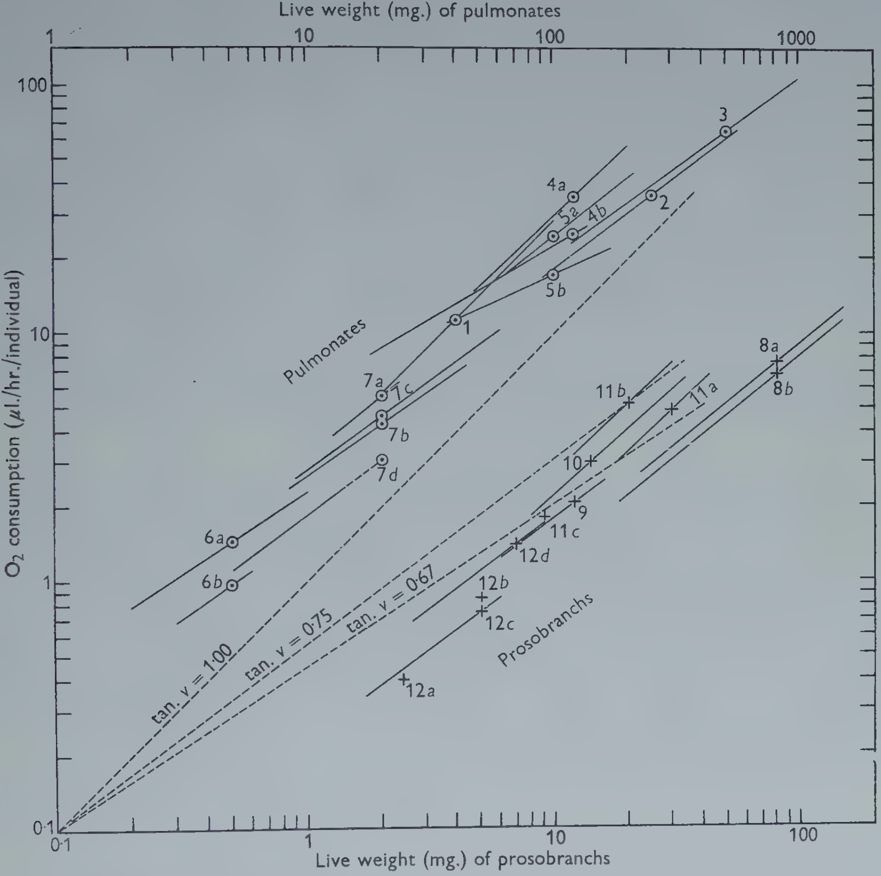

Fig. 4. Oxygen consumption in relation to live weight of the standard individuals of all the gastropods studied. Experimental temperature 18° C. The regression lines, found graphically, of most of the experiments are drawn. The scale of the live weight of the pulmonates (the top abscissa) is ten times as large as the scale for the prosobranchs (bottom abscissa), and the two groups are accordingly divided. *Pulmonates:* 1. *Physa fontinalis* (experiment carried out in April; the value corrected 10 % for starvation). 2. *Myxas glutinosa* (May). 3. *Lymnaea auricularia* (May, experiment at 13°, corrected to 18° C.). 4. *Lymnaea pereger* (*a*, June; *b*, August). 5. *Lymnaea palustris* (*a*, June; *b*, August). 6. *Acroloxus lacustris* (*a*, May–June; *b*, September–October (Berg, 1952)). 7. *Ancylus fluviatilis* (*a*, June and *b*, October, both from the river, Funder Aa. *c*, August and *d*, December, both from the lake, Rørbæk Sø; the last-mentioned experiment carried out at 13° and corrected to 18° C.). *Prosobranchs:* 8. *Bithynia tentaculata* (*a*, June; *b*, October). 9. *Bithynia leachi* (September, the value corrected for starvation). 10. *Valvata piscinalis* (September). 11. *Theodoxus fluviatilis* (*a*, from fresh water, June; *b*, from brackish water, interpolated; *c*, from brackish and fresh water, July, experiment at 19°, corrected to 18° C. (Lumbye, 1958)). 12. *Potamopyrgus jenkinsi* (*a*, June, from fresh water, interpolated; *b*, June, from brackish water; *c*, July, from fresh water, experiment at 19°, corrected to 18°; *d*, July, from brackish water, experiment at 19°, corrected to 18° C. (Lumbye, 1958)).

individuals in the experimental animals, since all of them were beyond the juvenile stage. The variation, however, is not in accordance with Bertalanffy's conception of three metabolic types.

With reference to the observation that species in the course of their ontogenetic development pass through successive stages characterized by regularly changing values of b (= slope of regression line), Zeuthen (1955) has already surmised that there are many more 'metabolic types' which should be related to phenomena of growth than the three (surface, weight, or intermediate) suggested by Bertalanffy.

As mentioned above, the slopes b of the regression lines of *Lymnaea palustris* and *L. pereger* were greater in June than in August. In *Ancylus fluviatilis* it was found earlier that the oxygen consumption itself is greater during the period of reproduction than during other seasons (Berg *et al.* 1958). It is reasonable, therefore, to suppose that the same applies to the two *Lymnaea* species as well. If this is the case the seasonal variation of b may be caused by a comparatively greater increase during the season of reproduction (June) of the oxygen consumption of the larger individuals with a more vigorous reproduction than of the smaller ones, which, though mature, produce fewer eggs. In August, when reproduction declines, this difference of the oxygen consumption is not so clearly manifested and b therefore decreases.

(*b*) *Interspecific variation*

In Fig. 4 the oxygen consumption at 18° C. of the prosobranchs and pulmonates is shown in relation to the live weight of the species. The regression lines of all experiments are drawn graphically as they are found in experiments similar to those depicted in Fig. 3; the respiration of the standard individuals is shown by means of crosses and circles, respectively. The scale of the top abscissa (of pulmonates) is ten times as large as the scale of the bottom abscissa (of prosobranchs) in order to separate the two groups. The projection of the regression lines on the abscissa indicates the size variation of the experimental animals concerned.

Fig. 4, in addition to new experiments, shows the results of earlier experiments on *Acroloxus lacustris* (Berg, 1952, fig. 8*b* corrected to 18° C. from the experimental temperature 16° C.), experiments on *Ancylus fluviatilis* (Berg, 1953), and also on *Theodoxus fluviatilis* and *Potamopyrgus jenkinsi* from fresh and brackish water according to Lumbye (1958), who carried out his experiments in our laboratory and with the same methods. The species were taken from ecologically very different biotopes (cf. p. 690). It will be noted that the live weight of the standard individuals varies from 2·5 to 500 mg.

The following slopes b of the regression lines were found for prosobranchs:

Bithynia tentaculata	0·81	*Theodoxus fluviatilis*	0·95
B. tentaculata	0·80	*T. fluviatilis*	0·94
B. leachi	0·74	*Potamopyrgus jenkinsi*	0·73
Valvata piscinalis	0·89	*P. jenkinsi*	0·73

Thus it is seen that some values of b have nearly the value 0·67 required by the surface law, but others are markedly higher, up to 0·95.

According to Krywienczyk (1952*a*, cf. Bertalanffy, 1957) the prosobranchs should have an oxygen consumption proportional to the surface, i.e. proportional to $w^{\frac{2}{3}}$, where w is the weight of the species. Our experiments have shown that in the case of *Bithynia leachi*, and in *Potamopyrgus jenkinsi* this may nearly be so. But in other species oxygen consumption may vary much more in relation to weight, being sometimes nearly proportional to weight.

In pulmonates the oxygen consumption in relation to weight (Fig. 4, at the top) also varies. The slope of the regression line, b, is found to be in the case of

Physa fontinalis	1·00	*Acroloxus lacustris*	0·67
Myxas glutinosa	0·75	*A. lacustris*	0·70
Lymnaea auricularia	0·72	*Ancylus fluviatilis*	0·80
L. pereger	0·94	*A. fluviatilis*	0·75
L. pereger	0·59	*A. fluviatilis*	0·73
L. palustris	0·76	*A. fluviatilis*	0·70
L. palustris	0·45		

Thus the oxygen consumption of pulmonates in relation to weight varies from $b = c.\ 0{\cdot}45$ to $b = c.\ 1{\cdot}00$, i.e. between less than proportional to surface and proportional to weight.

According to Bertalanffy (1957) the relation between metabolic rate and body size for pulmonates varies in such a way that the respiration in some cases is proportional to the body surface (according to experiments by Brand, Nolan & Mann, 1948) and in some other cases is intermediate, i.e. proportional to more than 2/3 but less than 3/3 power of the weight. The last-mentioned instances include species of *Lymnaea*, *Planorbis* and *Isidora* according to experiments by Bertalanffy & Müller (1943), Füsser & Krüger (1951) and Krywienczyk (1952*b*). Thus the experiments reported in this paper are in agreement with the observations of other workers except where they have shown respiration to be proportional to body weight. Experiments with *Lymnaea auricularia* by Krywienczyk (1952*b*) have possibly also shown respiration proportional to body weight.

(*c*) *Oxygen consumption of the freshwater snails as a group*

Fig. 4 also calls for comment on the oxygen consumption of the freshwater snails regarded as a group, a unity. It will be seen that the oxygen consumption of all the standard individuals together is depicted as a belt showing only a slight dispersion. The width of the belt, the dispersion, includes a seasonal variation of some of the species. In spite of this it is characteristic of the belt, formed by the respiration values of the standard individuals, that it is narrow, i.e. the freshwater snails examined have a fairly uniform respiration.

The relation of the oxygen consumption to the size of the freshwater snails as a group seems, according to Fig. 4, to be expressed by a regression line with a slope just below 1·0 and at any rate greater than 0·75. This fact is of course not inconsistent with the above-mentioned result, that most of the species have different values for the constant b. Furthermore, there does not seem to be any difference

between the two groups investigated, pulmonates and prosobranchs, with regard to the slopes.

It seems noteworthy that the slope of the regression line of the freshwater snails as a group does not fit in with the common regression line of poikilotherms and homiotherms described by, e.g. Hemmingsen (1950, p. 11) and Zeuthen (1953, p. 3). The difference is supposed to be important for the understanding of the phylogeny of the group in relation to physiology. In this connexion it should be added that the results found by us so far only apply to mature animals, and among these the usual phenomenon of larger species having a lower rate of respiration per unit weight has been observed only to a very small extent. If immature stages were investigated the results might be different.

It may be mentioned incidentally that *Pisidium* sp. has an oxygen consumption of *c.* 0·4 μl./hr./individual of 2 mg. at 18° C., and this value falls also within the belt formed by the respiration values of the freshwater snails.

OXYGEN CONSUMPTION IN RELATION TO THE OXYGEN CONTENT OF THE WATER

(*a*) The main purpose of this study was to see whether or not the freshwater snails were able to maintain their oxygen consumption with decreasing oxygen content of the water. If the oxygen consumption falls, it is interesting to see if the fall sets in as soon as the oxygen concentration decreases, or only after the oxygen concentration has reached a low level.

As before, the oxygen consumption here recorded was an active respiratory rate. Concerning this Fry (1957) writes as follows with reference to fishes: 'Any reduction of the oxygen content below the level where the active metabolic rate begins to be restricted is probably unfavourable to the species concerned. From the ecological point of view this "incipient limiting level" (the critical level under conditions of activity) can be taken as the point where the oxygen content begins to be unsuitable. The level of the beginning of respiratory dependence as an index of water quality was probably first proposed by Lindroth (1940) and formalized by Fry (1947)....'

The series of experiments reported below may serve to demonstrate the incipient *limiting* or *critical level of oxygen supply for freshwater gastropods*.

(*b*) Methods. All experiments on the oxygen consumption at a certain oxygen content of the water were carried out in the way described by means of five to six determinations (p. 691). The first experiment of a series began immediately after return to the laboratory with the animals in lake or pond water saturated with atmospheric air. In the following experiments water in equilibrium with nitrogen mixtures containing, e.g. 18·7 %, 16·2 %, 12·8 %, 10·0 %, 7·4 %, 4·7 % and 2·7 % of oxygen was used. The experimental time was most often 1 hr., and the experiments were carried out one after another during the same day. During each experiment the oxygen concentration of the water decreased from the above-mentioned oxygen percentages to about two-thirds of these.

In Fig. 5 a series of consecutive experiments on the oxygen consumption in relation to varying oxygen supply is shown; initial and final oxygen concentrations

of each experiment are indicated by means of arrows, and the average concentration by means of a cross. A curve through the mean values shows the result. The mean of an initial and final oxygen concentration is accepted as a useful approximation to the concentration of oxygen in which the animals have respired. The curves in Figs. 6 and 7 are drawn in a similar way through the means of the oxygen concentrations, leaving out the initial and final values of concentration.

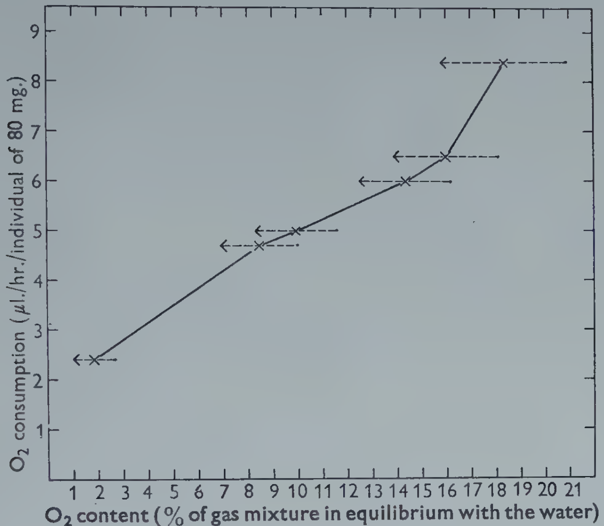

Fig. 5. Oxygen consumption of *Bithynia tentaculata* at 18° C. in relation to oxygen content of water. The arrows show the decrease of oxygen in the respiration bottles during the various experiments. The curve shows the oxygen consumption and the mean oxygen concentration during the experiments.

In Fig. 6 the standard live weights of the species, the experimental temperatures and the season are indicated after the names of the snails.

(*c*) The results of the series of experiments shown in Fig. 6 give occasion for the following remarks on the various species.

Lymnaea auricularia. The characteristic feature is that the species is nearly able to maintain its oxygen consumption in relation to decreasing oxygen concentration of the water down to about 11 %, but at lower concentrations the uptake decreases distinctly.

Myxas glutinosa. The usual oxygen consumption is maintained down to a concentration of about 12 % oxygen, but the consumption decreases at lower oxygen concentrations, especially below a content of *c.* 6 % O_2.

Lymnaea pereger. Immediately after decrease of the oxygen content of the water the oxygen uptake decreases; below a content of *c.* 8 % of oxygen the decrease of uptake is marked.

L. palustris. The oxygen consumption decreases at once with declining oxygen content, but seems to increase again; below 12–13 % of oxygen content of the water the decrease of uptake is fairly regular.

Bithynia tentaculata. The oxygen consumption decreases distinctly as soon as the oxygen supply declines.

Physa fontinalis. Even if the oxygen consumption first decreases a little the species is able to maintain the normal uptake at a concentration of 13–14% of oxygen; at a lower oxygen concentration the uptake decreases slightly and below a concentration of *c.* 6% of oxygen it decreases distinctly.

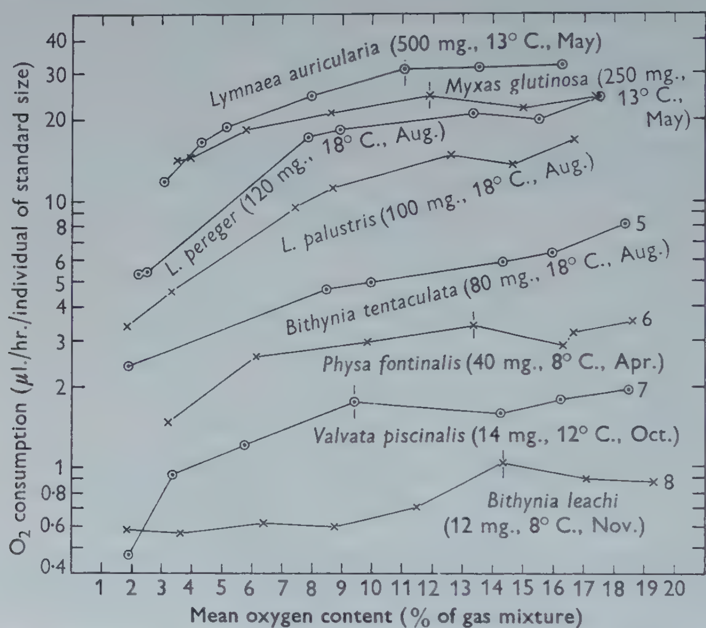

Fig. 6. Oxygen consumption in relation to oxygen content of the water. The consumption is shown along the ordinate in μl. per hr. per live weight (mg.) of the various standard individuals. The oxygen content is shown along the abscissa as the oxygen percentage of a gas mixture in equilibrium with the water, the values being the mean during the experiments (cf. the text). Vertical lines crossing the curves indicate approximately the critical points of oxygen supply.

Valvata piscinalis. It is nearly able to maintain its oxygen consumption with declining oxygen content of the water down to 9–10%; after that a distinct decrease in uptake is found.

Bithynia leachi. The consumption is maintained, or it has even increased a little, till the oxygen concentration has declined to 13–14%; but below this concentration the uptake decreases. The respiration at low oxygen percentages, however, is comparatively great, about two-thirds of the oxygen consumption in air-saturated water.

Summing up it may be said:

(*a*) A critical point of oxygen supply in the sense of Fry has been found in *Lymnaea auricularia* (*c.* 11% O_2), *Myxas glutinosa* (*c.* 12% O_2), *Physa fontinalis* (13–14% O_2), *Valvata piscinalis* (9–10% O_2) and *Bithynia leachi* (14–15% O_2). But the critical point is not very pronounced.

(*b*) In some other species oxygen consumption decreases immediately in response to a declining oxygen supply: *Lymnaea pereger*, *L. palustris* and *Bithynia tentaculata*. Among these species *Lymnaea pereger* and *L. palustris* increase uptake once more at low oxygen levels, but not to the initial value. In these cases there seems to be some reaction, perhaps increased activity, to the reduction of oxygen consumption. A similar increase of oxygen consumption following a moderate decrease is also found in *Myxas glutinosa* and *Physa fontinalis*, and in these cases the increase is so great that the oxygen consumption attains almost the initial value.

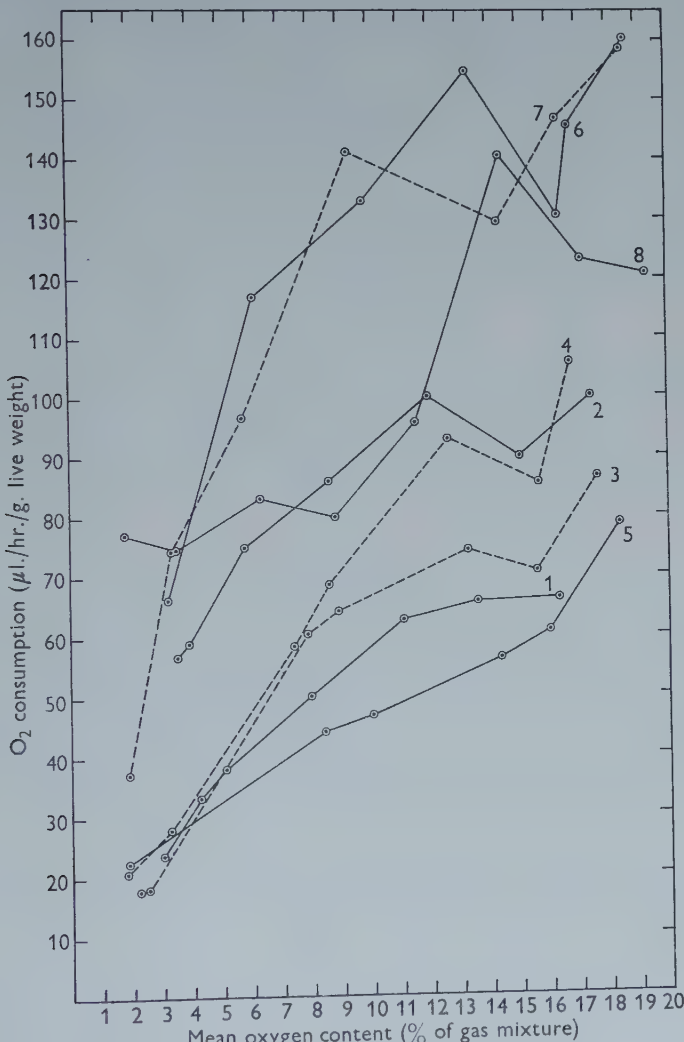

Fig. 7. Oxygen consumption in relation to the decreasing oxygen content of the water. The same results of experiments as indicated in Fig. 6, but the consumption is here shown per gram live weight at 13° C. For further explanation see Fig. 6 and the text. 1. *Lymnaea auricularia*. 2. *Myxas glutinosa*. 3. *Lymnaea pereger*. 4. *L. palustris*. 5. *Bithynia tentaculata*. 6. *Physa fontinalis*. 7. *Valvata piscinalis*. 8. *Bithynia leachi*.

(*c*) In some species the decrease in oxygen consumption in response to a decreasing oxygen supply is not gradual, but shows a steep fall below certain values of the oxygen content: For *Myxas glutinosa* at *c.* 6 % O_2, for *Lymnaea pereger* at *c.* 8 % O_2, and for *Physa fontinalis* at *c.* 6 % O_2.

(*d*) The only species able to maintain a comparatively high oxygen consumption at low oxygen supply is *Bithynia leachi.*

In order to make certain comparisons the results shown in Fig. 6 are computed as oxygen consumption per gram (instead of per standard individual) and at 13° C., which is the temperature midway between the experimental temperatures actually used. The values of oxygen consumption calculated in this way are depicted in Fig. 7.

Fig. 7 shows that when the oxygen supply is abundant the two small species *Valvata piscinalis* and *Bithynia leachi* have, as might have been expected, a great oxygen consumption. The same applies to *Physa fontinalis*, which also is fairly small and in addition is usually more active than the other species examined. Furthermore Fig. 7 shows that at a low oxygen content of the water, below *c.* 4–5 % O_2, *Bithynia leachi* is the only species able to maintain a great and steady oxygen consumption (*c.* 75 μl./g./hr.); among the other species the oxygen uptake is lower under these circumstances and also decreases distinctly in consequence of declining oxygen supply.

The incipient limiting or critical point of oxygen supply is seen very clearly in Fig. 7 for *Physa fontinalis* (13–14 % O_2), *Valvata piscinalis* (9–10 % O_2), *Bithynia leachi* (14–15 % O_2), *Myxas glutinosa* (*c.* 12 % O_2) and, less pronounced, *Lymnaea auricularia* (*c.* 11 % O_2). Altogether the critical points of oxygen supply are found to occur from about 9 to 15 % O_2, that is from just under half to about threequarters of air-saturation.

DISCUSSION

Oxygen consumption and oxygen supply related to the ecology of the species

If an organism is able to maintain its usual oxygen consumption until the oxygen supply falls to a certain low critical value, this must be favourable to the organism. If the oxygen consumption decreases as soon as the oxygen supply diminishes it must be unfavourable, because one or more physiological functions of the organism must then have been depressed. But the latter way of reaction need not be so decisive for the organism that it cannot for that reason exist in a given locality with a more or less bad oxygen supply. Perhaps the species merely does not thrive so well, e.g. its growth, the extent of its egg production, etc. are reduced. Thus the difference between two species, one of which has a critical level (point) of oxygen supply while the other has not, does not necessarily mean such a difference in an ecological respect that it has a decisive influence on their existence in localities poor in oxygen, i.e. on their distribution. There may be other differences, e.g. in food requirements, which are more important for the distribution. No absolute correlation between the observed respiratory characteristics of the freshwater snails and their distribution in nature can therefore be expected. Nor was such a correlation found in the case of the snails investigated.

On the other hand, respiratory curves with or without a critical point no doubt express an essential physiological difference between the species concerned. Under bad respiratory conditions the species having a critical point at a low oxygen supply must be expected to thrive better than the species showing a decreasing oxygen consumption with decreasing supply.

At a low oxygen content of the water the oxygen consumption of all the species investigated, with the exception of *Bithynia leachi* (p. 704), is reduced very considerably. This physiological quality of *B. leachi* is probably of importance. In Esrom lake the specimens used were collected both in the littoral zone between vegetation and on gravel, where the water is undoubtedly rich in oxygen, and also in a yachting harbour on a muddy bottom, which smells of hydrogen sulphide and where the water must be very poor in oxygen; there the shells are dark with ferrous sulphide. Under these conditions the snails' ability to maintain a great oxygen consumption is probably an advantage.

Freshwater molluscs as a whole seem to possess a considerably greater physiological adaptability than, e.g. marine molluscs. The comparatively small differences as to respiration of the freshwater snails examined are probably connected with their ability to live together in ecologically very different places. Nevertheless, for some of them the oxygen conditions in a certain locality may be of decisive importance.

From E. Frömming, 'Biologie der mitteleuropæischen Süsswasserschnecken' (1956) it seems that small species do not have a shorter life span than most of the larger ones, i.e. the growth rate of the former must be considerably smaller than that of the latter. In localities where the oxygen content of the water is low during a shorter or longer period small species are especially common, i.e. those which have a small growth rate. *Valvata* and *Bithynia*, for instance, occur at fairly great depths in the eutrophic lakes and in other places with bad respiratory conditions, whereas the large species of *Lymnaea* occur especially near the surface of ponds or at the shores of lakes and rivers. A similar example is represented by the Danish *Pisidium* species, among which *Pisidium amnicum* (O. F. Müller) attains by far the greatest size. And just this species seems to be restricted to localities with fairly great water movements, i.e. localities having favourable oxygen conditions. The occurrence of the most suitable food must, of course, also be important for the growth and occurrence of the species.

Seasonal variation of the oxygen consumption of freshwater snails

It was shown earlier that the variation in the oxygen consumption of *Ancylus fluviatilis* in the course of the year is great. Measured at the same temperature the oxygen consumption is about 1·3 times to nearly twice as great in spring and summer as in autumn and winter. The increase of the oxygen consumption during the reproductive period is regarded as an expression of the sexual activity (Berg *et al.* 1958).

A similar seasonal variation was shown for *Acroloxus lacustris* (Berg, 1952, fig. 8*b*). If the demonstrated oxygen consumption is converted from the experimental tem-

perature 16° to 18° C. by means of the relation also shown to exist between the respiration of this limpet and the temperature the following values are found:

May–June (1949): 1·44 μl. oxygen consumption/hr./individual of 5 mg.
September–October (1950): 0·97 μl. oxygen consumption/hr./individual of 5 mg.

Thus the oxygen consumption of *A. lacustris* in spring and early summer is found to be about 50% greater than in autumn (the measurements were carried out with the same methods).

Some of the experiments mentioned in this paper indicate also the existence of a seasonal variation in some other species. Thus in the case of *Lymnaea pereger* the following values were found at 18° C.:

June: 35·2 μl. oxygen consumption/hr./individual of 120 mg.
June: 30·6 μl. oxygen consumption/hr./individual of 120 mg.
August: 25·0 μl. oxygen consumption/hr./individual of 120 mg.

The two values from June are about 40% and 22% greater than the value from August. It is therefore reasonable to suppose that there exists a seasonal variation in this species.

In *Lymnaea palustris* the following values were found at 18° C:

June: 24·6 μl. oxygen consumption/hr./individual of 100 mg.
August: 17·2 μl. oxygen consumption/hr./individual of 100 mg.

Here also there is a distinct seasonal variation. The oxygen uptake in June is 43% greater than in August.

In *Bithynia tentaculata* at 18°:

July: 7·6 μl. oxygen consumption/hr./individual of 80 mg.
August: 8·4 μl. oxygen consumption/hr./individual of 80 mg.
October: 6·8 μl. oxygen consumption/hr./individual of 80 mg.

The value from July is *c.* 12% greater than the value from October, and thus this also may indicate the existence of a seasonal variation.

The experiments on the other snails were carried out in the same season of 1957 and not distributed over several months. Hence it is not possible to say anything about the seasonal variation of the respiration of these species. But on the basis of the above-mentioned examples it must be presumed that a seasonal variation in oxygen uptake is fairly common in Danish freshwater snails, and the variation may be very extensive, at least up to 100% of the lowest value. Seasonal variation is, therefore, a quality which must be considered in intra- and interspecific comparisons of the physiology of snails.

SUMMARY

1. The oxygen consumption of some Danish freshwater snails was studied in relation to varying periods of starvation, varying temperatures, weight of animals and oxygen content of the water. The observed respiration is a moderately active metabolism, not a basal one.

2. In the case of *Lymnaea palustris* and *Bithynia leachi* a distinct decrease of oxygen consumption has been found in the period 1–24 hr. after collecting; the decrease is supposed to be caused by starvation. In similar experiments *Lymnaea pereger*, *Myxas glutinosa*, *Bithynia tentaculata*, *Valvata piscinalis* and possibly *Physa fontinalis* and *Lymnaea auricularia* show only a small decrease (or no decrease) of oxygen consumption.

3. During a gradual increase of the temperature (*c.* 1° C. per hr.) the snails increase their oxygen consumption by 65–90% of the increase expected from Krogh's curve. In the case of *Myxas glutinosa* and *Physa fontinalis* the increase of respiration was nearly the same as that found by Krogh for other animals.

4. The relation of oxygen consumption to body size (live weight) is not a fixed, unchangeable quantity characteristic of every species, but may vary seasonally. A tentative explanation of this variation is given.

5. The oxygen consumption in relation to body size has also an interspecific variation. In prosobranchs the slopes b of the regression lines in a logarithmic co-ordinate system have in some cases nearly the magnitude 0·67 required by the surface law, but others are higher, e.g. *c.* 0·95. In pulmonates the relation varies as much as from $b = c.$ 0·45 to $b = c.$ 1·00, i.e. between less than proportional to surface and proportional to weight.

6. The oxygen consumption of the freshwater snails in relation to the sizes of the standard individuals is depicted in a logarithmic co-ordinate system as a belt showing only a slight deviation (Fig. 4, p. 697), i.e. the snails regarded as a whole have a fairly uniform respiration. The regression line of oxygen consumption to sizes of the standard individuals seems to be expressed by a regression line with a slope just under 1·0.

7. Experiments on oxygen consumption in relation to oxygen content of the water have shown that some species (*Lymnaea auricularia*, *Myxas glutinosa*, *Physa fontinalis*, *Valvata piscinalis* and *Bithynia leachi*) are able to maintain their consumption with decreasing oxygen content of the water to a critical point of oxygen supply. But in some other species (*Lymnaea pereger*, *L. palustris* and *Bithynia tentaculata*) oxygen consumption decreases immediately in response to a declining oxygen supply.

8. In some freshwater snails (*Myxas glutinosa*, *Lymnaea pereger*, *Physa fontinalis*) the decrease in oxygen consumption in response to a decreasing oxygen supply is not gradual, but shows a steep fall below certain low values of the oxygen content. The only species able to maintain a comparatively high oxygen consumption at low oxygen supply is *Bithynia leachi*.

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SODIUM AND POTASSIUM IN THE ENDOLYMPH AND PERILYMPH OF THE STATOCYST AND IN THE EYE OF *OCTOPUS*

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INTRODUCTION

In the majority of invertebrates the statocyst consists of a single sack; only in the octopod cephalopods is there one sack inside another, so that one can distinguish endolymph and perilymph as in the vertebrate labyrinth. Even in the decapod cephalopods (squids and cuttlefishes) no inner sack is present. The significance of the two sacks has yet to be determined, but it is clearly of interest to discover the composition of the fluids in the two compartments, and in particular whether the endolymph contains high potassium and low sodium concentrations, as in mammals (Smith, Lowry & Wu, 1954; Citron, Exley & Hallpike, 1956; Rauch & Köstlin, 1958). At the same time we have taken for comparison a few samples of fluid from the anterior and posterior chambers of the eye, and from the blood.

The endolymphatic and perilymphatic spaces both contain colourless fluids. The perilymph is crossed by numerous fibrous strands carrying blood vessels to the inner sack. These give it a remarkable resemblance under the microscope to the perilymphatic space of vertebrates. In fixed sections the liquid differs in appearance from blood and contains no cells, but often a coarse precipitated material. The endolymph differs in appearance from both blood and perilymph. In sections it also shows a precipitate but no cells (Young, 1959).

There is no obvious special apparatus for producing the perilymph. The outer wall of the statocyst is formed of a vascular type of cartilage, not lined by an epithelium. Into the endolymphatic space there opens a canal, representing the remains of the embryonic communication with the ectoderm and known as Kölliker's duct. This is ciliated and might serve for either production or absorption of endolymph. There are muscle fibres in the wall of the sack and in life the contents seem to be under a small pressure.

Robertson (1949, 1953) measured the sodium and potassium concentrations in the blood and eye fluids of several cephalopods, but so far as we are aware, these analyses have not previously been performed on the body fluids of *Octopus vulgaris*, nor on the statocyst fluids of any cephalopod.

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METHODS

Collection of samples

A single statocyst contains volumes of 30 μl. of perilymph and 15 μl. of endolymph, or even more in larger specimens. However, in order to obtain uncontaminated specimens, much smaller samples were used, in the range of 0·1–3·5 μl., making 'ultra-micro' methods of analysis necessary. Fluids were collected in capillary tubes of borosilicate glass. These were cleaned in hot chromic acid fluid for 1 hr. and then washed with running tap water and distilled water. Distilled water was drawn through each tube, followed by acetone. When dry the tubes were drawn to tip diameters of 10–50μ and stored in container tubes.

Octopuses were killed by decapitation, without previous anaesthesia. Each statocyst in turn was then immediately exposed by cutting away first skin and then cartilage with a sharp blade under a low-power microscope. When the cartilage had been cut so as almost to open the perilymph its surface was wiped with filter paper and the blade itself was dried. A further cut then opened the perilymph and a little fluid escaped.

A pipette, previously prepared, was then advanced with a simple micro-manipulator into the cavity and fluid was sucked into it with a suction pump, the flow being regulated with a valve under manual control. Considerable suction was necessary to collect the fluid and pipettes with finer tips ($< 10\mu$) frequently became blocked, presumably by the strands in the perilymph. Tips of 50μ were found to be more convenient.

After a small sample of perilymph had been collected the rest of it was removed by suction with a coarse pipette. In this way a dry cavity was obtained. A fresh pipette was then moved towards the turgid endolymphatic sack and with a sharp advance was made to enter it. By immediate suction a small sample could then be obtained directly from the sack itself. However, the fluid rapidly escaped from the sack and collected in the emptied perilymphatic space. In most experiments some of this fluid was also collected (as a separate sample) and was found not to differ from that obtained immediately after puncture. It is therefore thought that there was no serious contamination of the endolymph samples by the remains of perilymph, either on the outside of the sack or in the cavity.

The samples from the first statocyst opened were obtained within 15 min. of death. The second statocyst was then treated in the same way.

Samples of the fluids from the anterior and posterior chambers of the eye were obtained by similar methods, after opening each with scissors and inserting pipettes. These fluids are also colourless and transparent in life. The anterior fluid is in virtual communication with the seawater through the overlapping pseudo-corneal folds (Boycott & Young, 1956).

Blood was obtained from the ventricle or the orbital sinus.

Analytical methods

The methods of handling each sample of fluid and measuring its volume were based on those described by Exley (1956), with the following modifications. The sample (0·1–3·5 μl.) was discharged from the collecting capillary into the centre well of a 4·5 cm. diameter Conway unit. The unit rested on the base of a 500 ml. beaker, which was inverted in a 1500 ml. beaker to prevent draughts. The outer well of the unit, and the space between the beakers, were filled with wet tissue paper. Between samples, the outer beaker was covered with a Petri dish lid to preserve a moist atmosphere. The inside of the smaller beaker was blackened to make the sample more visible. The Conway unit was not silicone-treated; the centre well was wiped clean with moist and then with dry tissue paper between each sample. With minimum delay the sample was drawn into a capillary pipette, the surface (including the tip) was wiped with tissue paper, and the position of the meniscus was marked. The sample was quantitatively transferred into 1·0 ml. of 0·0025 M-$Li_2SO_4.H_2O$ in a 6 ml. stoppered polythene specimen tube (A. Gallenkamp and Co., London, E.C. 2); the capillary was rinsed three times by drawing the lithium sulphate solution up above the mark and blowing it out again. After being washed and dried, the pipette was refilled to the mark with a standard solution containing 0·518 M-NaCl and 0·016 M-KCl (i.e. approximating to seawater with respect to sodium and potassium concentrations). This solution was quantitatively transferred into a second 1·0 ml. of 0·0025 M-Li_2SO_4. Both polythene tubes were stoppered and shaken. The solutions were aspirated in a lithium internal-standard flame photometer (Amoore, Parsons & Werkheiser, 1958). By fitting a no. 16 hypodermic needle to the atomizer the aspiration rate was decreased to 0·5–0·6 ml./min., permitting two readings for sodium and two for potassium on each 1·0 ml. sample.

Calibration curves were prepared from the flame photometer readings obtained when solutions containing known concentrations of sodium and potassium in 0·0025 M-Li_2SO_4 were aspirated. The volume of the octopus fluid sample was calculated from the quantity of sodium found after refilling the pipette with standard saline of known molarity (0·518 M-NaCl) as described above. The concentrations of sodium and potassium in the sample were calculated from the flame photometer readings and the determined volume.

In order to determine that there was no evaporation of the sample during analysis, ten samples of standard saline (0·518 M-NaCl) ranging in volume from 0·3 to 4 μl. were delivered from an ultra-microburette into the Conway unit, drawn up into capillary pipettes and the sodium concentration determined as described above. The mean concentration of sodium found was 519 m.-equiv./l.; S.D. ± 16. Within these volume limits the accuracy was independent of the sample size. The coefficient of variation for a single flame photometric determination of sodium or potassium was 1·6 % for aspirated solutions containing between 0·04 and 2·0 m.-equiv./l., and 5 % for solutions containing between 0·012 and 0·04 m.-equiv./l.

RESULTS

The concentrations of sodium and potassium (mean ± S.D.) found in the body fluids of *Octopus vulgaris* are summarized in Table 1. The perilymph and endolymph samples were obtained from the statocysts of seven octopuses. In all cases but one, only a single sample from the appropriate part of each statocyst was analysed; in that one case the figure used in the statistical analysis was the mean of the determinations on the two samples from the same part of the same statocyst. The eye fluids came from three octopuses, each sample being obtained from a different anterior or posterior chamber. The results of the blood analyses are those obtained from a single sample from the ventricle of one octopus, and the mean of three results on samples from the orbital sinus of a second octopus.

Table 1. *Concentrations of sodium and potassium in statocyst, eye and blood of* Octopus vulgaris

Fluid analysed	No. of samples	Volume of samples		Sodium concn. mean ± S.D. (m.-equiv./l.)	Potassium concn. mean ± S.D. (m.-equiv./l.)	Ratio Na^+/K^+
		Range (μl.)	Mean (μl.)			
Perilymph	10	0·12–3·44	1·17	555 ± 32	17 ± 6	33
Endolymph	12	0·10–1·23	0·38	601 ± 46*	20 ± 3	30
Anterior chamber	3	0·31–1·44	0·85	614 ± 68	19 ± 2	32
Posterior chamber	3	0·58–1·69	1·04	603 ± 12	26 ± 11	23
Blood	2	0·23–1·52	0·67	525 ± 3	30 ± 2	17
Seawater†	—	—	—	559	11·9	47·0

* One low value of 182 m.-equiv. Na^+/l. has been omitted from the statistical analysis.
† These values are based on an assumed chlorinity of 22·5 ‰ for the seawater at Naples (Robertson, 1953).

All the *Octopus vulgaris* fluids analysed had approximately the same concentration of sodium as the Naples seawater, but definitely higher concentrations of potassium. The blood in particular contained over twice the potassium concentration of the sea. Robertson (1949, 1953) showed that the bloods of *Eledone*, *Loligo* and *Sepia* contain about twice as high a concentration of potassium as does seawater.

There was no significant difference between the sodium content of blood and perilymph, but endolymph contained significantly more sodium than perilymph or blood ($P < 0·05$). There was no significant difference in potassium concentration between perilymph and endolymph, but both fluids had a lower concentration of potassium than the blood ($P < 0·05$).

The fluid from the posterior chamber of the eye had a potassium concentration near to that of blood, but this was not significantly higher than that in the anterior chamber of the eye. The fluids of the eye both had a similar sodium concentration to that of endolymph.

DISCUSSION

The sodium and potassium concentrations in octopus endolymph are markedly different from those in mammalian utricular and cochlear endolymph. Thus guinea-pig and human endolymph contain about thirty times the concentration of potassium found in the serum, and a correspondingly lower concentration of sodium (1/9 to 1/5 of that in serum), so that the usual high ratio of Na^+/K^+ found in most extracellular body fluids is substantially reversed in mammalian endolymph (Smith *et al.* 1954; Citron *et al.* 1956; Rauch & Köstlin, 1958).

It is not certain at which stage of vertebrate evolution this high concentration of potassium appeared in the endolymph. In elasmobranch fishes both endolymph and perilymph are said to have a high Na^+/K^+ ratio (Kaieda, 1930). The stria vascularis is probably closely involved in either the secretion or regulation of mammalian endolymph (Citron *et al.* 1956; Naftalin & Harrison, 1958). The stria first appears in a rudimentary form in some amphibians (Guggenheim, 1948). It would be interesting to know whether the production of a potassium-rich endolymph coincides phylogenetically with the emergence of the stria vascularis.

It is at present uncertain what connexion (if any) these ionic ratios have with the standing potentials between endolymph and perilymph, and the sensitivity of the hair cells (Davis, 1957). Whatever the significance of the presence of two fluids in *Octopus* may be, it is not connected with large differences in sodium and potassium concentrations. It therefore remains obscure why there should be two fluids in octopods whereas the related decapods, like other invertebrates, have only one sack.

The observations on the fluids of the eye confirm those of Robertson in showing that neither of these fluids is identical with seawater. In *Octopus*, as in the *Sepia* that he examined, there is a potential channel of communication between the anterior chamber and the sea. The pseudocorneal folds of *Octopus* divide the anterior chamber into inner and outer parts (Boycott & Young, 1956). The inner of these (from which the fluid here analysed was obtained) communicates with the outer (and thus with the sea) by a narrow channel. Nevertheless it has a potassium concentration higher than that of seawater, but lower than blood. At the back of this chamber is a specialized epithelium, the anterior chamber organ, connected with a series of strands, the subpedunculate tissue, which run into the optic lobes (Boycott & Young, 1956). This system may well play a part in regulating the composition of the fluid in the anterior chamber.

SUMMARY

1. The concentrations of sodium and potassium were measured in 0·1–3·5 μl. samples of the following body-fluids of *Octopus vulgaris*: perilymph and endolymph from the statocyst, anterior and posterior chamber fluid from the eye and blood.

2. All these fluids had approximately the same sodium concentration as the seawater, but slightly higher concentrations of potassium. The blood had the highest concentration of potassium, 30 m.-equiv./l., or over twice that in the sea. In the endolymph the potassium concentration was 20 m.-equiv./l.

We should like to thank Dr P. Dohrn and the staff of the Zoological Station at Naples for their help in obtaining the animals. We are grateful to Professor Sir Hans Krebs, F.R.S. for his interest in the work, and to Dr D. E. Hughes for advice. This work was aided by a grant from the Rockefeller Foundation.

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